

**Molecular Regulation of *Trypanosoma congolense*-Induced Proinflammatory
Cytokine Production in Macrophages and its Modulation by Diminazene
aceturate (Berenil)**

By

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ABSTARCT

African trypanosomiasis remains a major health problem to both humans and animals due to lack of effective treatment or vaccine to control the disease. Animal trypanosomiasis is considered one of the most important animal diseases affecting livestock production and agricultural development in sub-Saharan Africa. Although the use of trypanocides remains the most important method for controlling the disease in animals, the mechanisms of action of these compounds are still not completely known. The overall aim of this thesis is to decipher the molecular mechanisms involved in *Trypanosoma congolense*-induced cytokine production and how this is modulated by the trypanocide, Diminazene aceturate (Berenil).

First, we investigated the molecular and biochemical mechanisms of action of Berenil and whether Berenil, in addition to its trypanolytic effect, exerts a modulatory effect on the host immune response to *T. congolense*. Although it is known that *T. congolense* infection in mice is associated with increased production of pro-inflammatory cytokines by macrophages, the intracellular signaling pathways leading to the production of these cytokines remain unknown. Therefore in the second part of the thesis, I investigated the innate receptors and intracellular signaling pathways that are involved in *T. congolense*-induced pro-inflammatory cytokine production in macrophages. Next I further determined whether the inhibitory effect of Berenil on proinflammatory cytokine production in macrophages is specific to *T. congolense*.

I found that *T. congolense* infection leads to immune activation and increased production of proinflammatory cytokines. Berenil treatment significantly reduced serum levels of pro-inflammatory cytokines and altered the activation status of lymphocytes in the spleens and livers of infected mice. These results provide evidence that in addition to its direct trypanolytic effect, Berenil also modulates the host immune response to the parasite. Next, I showed that *T.*

congolense induces the production of IL-6, IL-12 and TNF- α in macrophages and this is dependent on phosphorylation of mitogen-activated protein kinase (MAPK) and signal transducer and activation of transcription (STAT) proteins in a TLR2-dependent manner. Deficiency of TLR2 leads to impaired cytokine production in macrophages, uncontrolled first wave of parasitemia and acute death in the otherwise relatively resistant mice. I further show that Berenil treatment downregulates *T. congolense*-induced cytokine production by affecting the phosphorylation of MAPK and STAT proteins. In the last part of the thesis, I showed that pretreatment of macrophages with Berenil dramatically suppressed LPS-, CpG- and Poly I:C-induced production of IL-6, IL-12 and TNF without altering the expression of Toll-like receptors (TLRs). Akin to its effect in *T. congolense* infection, Berenil significantly downregulated LPS-induced phosphorylation of MAPKs and STAT proteins and NF κ B p65 activity in macrophages *in vitro* and *in vivo*.

Collectively, the results from this thesis provide novel insights into *T. congolense*-induced activation of the innate immune system and modulation of host immune response by Berenil. In addition, they define the molecular and intracellular signaling events involved in the induction of proinflammatory cytokines by the parasite, and how Berenil acts to modulate these processes. These findings are significant and could help in developing newer and better therapeutic strategies against the disease in particular and inflammatory responses in general.

DEDICATION

This thesis is dedicated to the two heroes in my life: My father Kuriakose Kunnappillil and my husband Prince Joseph.

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ABBREVIATIONS

Acronym	Definition
AAT	Animal African Trypanosomiasis
ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of Variance
AP-1	Activating Protein-1
BCA	Bicinchoninic Acid
BMDC	Bone Marrow Derived Dendritic Cells
BMDM	Bone Marrow Derived Macrophages
CLR	C-type Lectin Receptor
CpG	Cytidine-phosphateguanosine
DEAE	Diethyl Aminoethyl cellulose
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECL	Electrochemiluminescence
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular Signal Regulated Kinase
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FcR	Surface receptor for the Fc portion of the Immunoglobulin
GAS	Interferon Gamma Activated Site
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor GPI
GPI	Glycosyl-phosphatidyl inositol
HAT	Human African Trypanosomiasis
HBSS	Hank's Balanced Salt Solution
HCT	Heamtocrit Centrifuge Technique
IFAT	Indirect Fluorescent Antibody Test
IFN- γ	Interferon Gamma
IFN- γ R	Interferon Gamma Receptor
IL-1R	Interleukin 1-receptor
iNOS	Inducible Nitric Oxide Synthase
ITAM	Immunosuppressor Tyrosine-based Activation Motif ITIM
ITIM	Immunosuppressor Tyrosine-based Inhibition Motif
JNK	c-Jun Terminal Kinase
kDNA	Kinetoplast DNA
LA	Latex Agglutination

LOXO1	Lectin-like Oxidized Low-density Lipoprotein Receptor
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MIF	Migratory Inhibitory Factor
MIP1	Macrophage Inflammatory Protein 1
MN	Mannose receptor
MyD88	Myeloid Differentiation Primary Response Protein 88
NFκB	Nuclear Factor κB
NLR	NOD-like Receptor
NO	Nitric Oxide
NOD	Nucleotide Binding Oligomerization Domain
NP40	Nonident P40
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PI3-K	Phosphatidylinositide-3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pattern Recognition Receptor
RHD	Rel Homology Domain
RIP2	Receptor Interacting Protein-2
RLR	RIG-like Receptor
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
RPMI	Rosewell Park Memorial Institute Medium
SIRS	Systemic Inflammatory Response Syndrome
SOCS-3	Suppressor of Cytokine Signaling-3
SOCS-1	Suppressor of Cytokine Signaling-1
STAT	Signal Transducer and Activator of Transcription Protein
TBST	Triss-Buffered Saline
TC	Trypanosoma congolense
TGF-β	Transforming Growth Factor β
Th1	T Helper 1
TIR	Toll Interleukin Receptor
TIRAP	TIR-domain Containing Adaptor Protein
TLR	Toll-like Receptor
TLR7	Toll-like Receptor-7
TLR9	Toll-like Receptor-9
TLR2	Toll-like Receptor-2

TLR4	Toll-like Receptor-4
TLR5	Toll-like Receptor-5
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAM	TRIF-related Adaptor Molecule
TREM	Triggering Receptor expressed by myeloid cells
TRIF	TIR Domain Containing Adaptor Inducing IFN- β
VSG	Variant Surface Glycoprotein
WCE	Whole Cell Extract
WHO	World Health Organization
WT	Wild Type
μg	Microgram

CHAPTER 1. INTRODUCTION

1.1 African trypanosomiasis

1.1.1 Introduction

African trypanosomes are flagellated protozoan parasites that cause disease in both humans and animals. The disease in humans are called Human African trypanosomiasis (HAT) or sleeping sickness and is considered as a neglected disease along with other parasitic diseases such as Leishmaniasis and Schistosomiasis [1]. Other than Malaria and Schistosomiasis, HAT is the 3rd significant contributor to the global burden of parasitic diseases [1]. The disease in humans is mainly caused by *Trypanosoma brucei rhodesiense* and *T. brucei gambiense*. Animal African trypanosomiasis (AAT) otherwise called as nagana, is caused by *T. congolense*, *T. brucei brucei* and *T. vivax*. *T. congolense* is the major species that primarily cause animal trypanosomiasis particularly in domestic livestock.

The disease is transmitted to the mammalian host by the bite of several species of tsetse fly vector belonging to the genus *Glossina*. The incidence of animal trypanosomiasis coincides with the distribution of their tsetse fly vectors. According to the 2014 WHO report, tsetse transmitted trypanosomiasis occurs in 36 sub-Saharan countries and covers almost 10 million kilometers of the African continent [2].

African trypanosomiasis has considerable economic and social importance and it is considered as one of the most important factors restricting economic development in Africa [3]. The disease threatens the health of about 70 million people and 50 million cattle. The recent WHO report indicates that due to continued control efforts, the number of cases has dropped for the first time in last 50 years. However sporadic cases and small outbreaks are reported increasingly in patients from non-endemic countries despite of the decline in the number of new cases of HAT

among Africans[4]. According to the 2014 WHO fact sheet, the estimated number of actual cases is 20000 and the estimated population at risk is 70 million people [2]. It is estimated that about 50 cases of HAT occur annually outside Africa and 94 cases of HAT were reported in 19 non-disease endemic African countries during the period of 2000 to 2010 [4, 5].

Although animal trypanosomiasis can occur in all domesticated animals, cattle are the main species affected due to the feeding preferences of tsetse flies. The disease adversely affects livestock production and farming and has a major impact on human and economic development. It is estimated that 3 million cattle die annually from the associated disease and the estimated economic loss in cattle production is about 4 billion US dollars per year [6].

Although the disease kills or disables hundreds of thousands of people and animals in endemic areas, the treatment for the disease is not satisfactory. Currently there are no vaccines available to prevent the disease in both humans and animals and the current treatment methods have several limitations and the development of drug resistance is a growing problem. All these have stimulated research into control or preventive regimens to save millions of humans and animals at risk of contracting African trypanosomiasis. Understanding the complexity of immunological mechanisms involved in resistance as well as in the pathogenesis of African trypanosomiasis will help in the development of effective prophylactic and therapeutic vaccines.

1.1.2 Classification

Trypanosomes are flagellated protozoan parasites belonging to the phylum Sarcomastigophora, class Zoomastigophora, order Kinetoplastida and family Trypanosomatidae. Members of the order kinetoplastida are elongate, slender and are characterized by the presence of an organelle called the kinetoplast. The kinetoplast is a mitochondrial DNA arranged in the form of a

network of thousands of topologically interlocked DNA circles [7]. The section Salivaria consists of groups of trypanosomes whose developmental cycle is completed within the salivary glands of the insect vector. Most members of this group are transmitted by several species of tsetse flies belonging to the genus *Glossina*, and are pathogenic to human or domestic animals. The development takes place in the salivary glands and therefore the transmission occurs by the bite of infected insect vector during a blood meal.

1.1.3 Morphology

Blood stream trypanosomes have an average length of 8-30 μm and are elongated and spindle shaped protozoa with a single nucleus and flagellum. The flagellum arises from the basal body at the posterior end and runs to the anterior end of the body. The attachment of flagellum along its length to the body pellicle forms the undulating membrane and afterwards it continues as a free flagellum at the anterior end. Kinetoplast DNA, which is an integral part of the mitochondrial system, is a unique organelle of trypanosomes. Within the salivarian trypanosomes, *T. congolense* are the smallest organisms with a length of 8-24 μm with a central nucleus and a marginal kinetoplast. *T. congolense* is usually monomorphic with a sluggish movement under the microscope and their undulating membrane is not well marked.

1.1.4 Life cycle:

1.1.4.1 Development in mammalian hosts:

When the trypanosome-infected tsetse fly takes a blood meal, it injects the metacyclic trypomastigotes to the dermal connective tissue of the mammalian host. They multiply extensively and produce a raised cutaneous swelling commonly referred to as chancre [8]. Eventually, the parasites enter the blood circulation through lymph vessels [8], and metacyclic trypomastigotes transform into bloodstream trypomastigotes. During the period of growth in the

blood stream, several morphological changes occur in the parasite. Different morphological forms vary from short, stumpy and usually non-dividing forms to long slender forms that undergo active cell division. Intermediate forms are the transition stage forms between long slender to stumpy forms. It has been suggested that for the cyclical development in the insect vector, the transformation from slender dividing forms to short stumpy non-dividing forms is necessary [9]. *T. brucei* are extracellular parasites and have the ability to penetrate the walls of capillaries and invade the interstitial spaces [10]. In contrast, *T. congolense* is an extracellular intravascular blood parasite that is unable to leave circulation.

1.1.4.2 Development in insect vectors:

The pathogenic salivarian trypanosomes are transmitted by several species of tsetse fly belonging to the genus *Glossina*. The tsetse fly ingests the parasite during a regular blood meal from an infected mammalian host. The bloodstream trypomastigotes transform into procyclic trypomastigotes in tsetse fly's midgut and parasites multiply by binary fission. During this period, the ingested parasites lose their variant surface glycoprotein (VSG) and undergo series of developmental and morphological changes. They migrate to the esophagus of the insect and then to the hypopharynx and transform into the epimastigote form. The epimastigotes migrate to the salivary gland and transform into metacyclic trypomastigotes. The parasite lives in the infected tsetse fly and this way the fly transmit the parasite for a considerable length of time.

1.1.5 Clinical disease and pathology

Although *T. congolense* and *T. brucei* infect cattle, sheep, horses, camels, swine and dogs, *T. congolense* is the most pathogenic trypanosome in cattle. The severity of trypanosome infection in animals depends on the virulence of the infecting strain and susceptibility of the host. The bite of tsetse fly results in the initial lesion or chancre at the bite site, which is characterized by local

erythemia, edema, heat and tenderness that disappears in 2 or 3 weeks. The initial clinical signs include intermittent fever, anemia, lymphadenopathy, weight loss and the animals become progressively emaciated [11]. Other clinical signs like intermittent fever, diarrhea, keratitis, lacrimation and appetite loss also have been reported. Death in trypanosomiasis is usually associated with severe anemia and circulatory collapse. The disease can cause abortion, premature birth and perinatal loss in animals [12]. Usually, trypanosomiasis is a chronic disease in susceptible animals with the syndrome lasting for months or years and is characterized by severe anemia and extreme emaciation. The trypanosome-infected animals have marked immunosuppression and are more prone to secondary bacterial infections [13].

1.1.6 The variant surface glycoprotein

The variant surface glycoprotein (VSG) is a dense and protective surface coat that covers the plasma membrane of African trypanosomes. It is about 12-15nm thick and is made up of millions of identical glycoprotein molecules and protects against complement-mediated lysis of the organism [14, 15]. Cross and his colleagues in 1975 isolated antigenically pure trypanosome variants and showed that their surface coat is composed of about 10^7 copies/ cell of 55 kD VSGs [14]. The VSG is essential for the blood stream trypanosomes, helping them to thrive in an unusually exposed niche being extracellular intravascular and are continuously exposed to the host immune system [16]. It forms a densely packed monolayer on the cell surface and helps to block antibody binding to other cell surface proteins. The VSGs exist in two clinical forms; the soluble (sVSG) and the membrane (mfVSG) forms. It has been shown that the VSG coats are stably associated with parasite plasma membrane and can be isolated as water-soluble glycoproteins (sVSG) after cell lysis [17]. When the VSG was isolated under denaturing

conditions, it retained the amphiphilic properties and this mfVSG was converted to sVSG during cell lysis by GPI- specific phospholipase C [18].

The VSG is attached to the external surface of the plasma membrane through glycosylphosphatidylinositol (GPI) [17]. The function of GPI is related to the release and shedding of VSG. Several studies have shown the presence of GPI anchors on more than 200 eukaryotic cell-surface proteins [19]. During the course of infection, the endogenous glycosylphosphatidylinositol-phospholipase C (GPI-PLC) cleaves the membrane form of VSG to release soluble VSG molecule from the membrane into blood and tissues [20]. The enzyme phospholipase C is abundantly present on the parasite and the VSG is cleaved by the phospholipase C, leaving the dimyristoglycerol compound (DMG) of the GPI on the membrane and VSG is released in the soluble form (sVSG) carrying the glycosyl inositol phosphate moiety of the GPI [21]. Studies have shown that African trypanosomes, under environmental or stress conditions are capable of activating the endogenous PLC resulting in the cleavage of VSG-GPI anchor, releasing sVSG into the circulation [21]. This cleavage eventually leads to Type1 immune activation as a result of generation of new trypanosome epitopes, characterized by the production of increased inflammatory cytokines [22].

1.1.7 Antigenic variation

Antigenic variation is an important immune evasion strategy that has evolved in certain pathogenic bacteriae, viruses and parasites. African trypanosomes are extracellular parasites and replicate in tissue fluids and blood stream and they are very well known for their extraordinary sophisticated ability to continuously change their variant surface glycoprotein coat. Although these parasites are continuously exposed to the host's immune attack, this ability to switch from one VSG on their surface to another helps them to effectively evade the host immune system by

being one step ahead of the host's immune response [23]. When the host is infected with trypanosomes, the host's immune system generates antibodies to the highly immunogenic VSG that helps in the elimination of most of the parasite population. However some parasites escape the antibody-mediated killing because by the time immune destruction is fully attained, some parasites have already started to express alternate VSG gene and thus the existing antibodies cannot bind. This cycling of parasites and antibody response continues throughout the infection resulting in undulating parasitemia and chronic infections in African trypanosomiasis.

It has been shown that the African trypanosomes contain about 1000 different VSGs and pseudo- VSGs [24]. Only one VSG is expressed at a time and the unexpressed VSGs are scattered among different chromosomes [25]. VSG switching involves DNA rearrangements replacing the old VSG and activation of another VSG expression site [24]. DNA switching involves two important mechanisms; first translocation of the previously silent VSG gene into transcriptionally active VSG expression site and therefore leading to the expression of a new VSG coat [23, 24]. The second mechanism involves differential transcription of the expression sites [23, 24].

Antigenic variation is certainly advantageous and is the most important evasion mechanisms to escape from the host immune response. However the exact molecular mechanisms involved in the switching of VSG gene expression is still not clear. Due to the complexity of the mechanisms involved in the VSG switching, it is highly unlikely to develop an effective vaccine against African trypanosomes.

1.1.8 Diagnosis

Diagnosis of African trypanosomiasis has problems, as the techniques for trypanosome detection are not sensitive enough and the clinical signs are not pathognomonic [26]. Even though there

has been significant improvement in the diagnosis, larger proportions of infections still remain undetected.

1.1.9.1 Microscopy and Mouse inoculation:

Diagnosis of trypanosomiasis is based on the presence of clinical signs but none of the clinical signs like anaemia, weight loss, abortion are pathognomonic to the disease. Most commonly used method for diagnosis is blood examination by light microscopy. Wet blood smears, Giemsa stained thin and thick blood smears and Buffy Coat Phase Contrast/ Dark Ground Techniques are used to examine for the presence of trypanosomes. The examination of fresh or stained blood films is modified by concentrating the blood by hematocrit centrifuge technique (HCT) or dark ground buffy coat technique. More infections were determined in the peripheral blood smears than the systemic blood films [27]. To detect *T. congolense* and *T. vivax* in the blood of cattle, the dark ground/ phase contrast buffy coat method has proven to be more sensitive than the hematocrit centrifugation technique [28]. On the other hand in *T. brucei* infection, mouse inoculation has been shown as the most sensitive method for parasite detection than the hematocrit centrifugation technique [28].

Freshly collected blood can be inoculated into laboratory rodents, later examined to see whether they have developed parasitemia. The choice of which technique to use in the field is determined by practical considerations. The lack of generators and centrifuges exclude the possibility of HCT and DG techniques whereas remote location prevents the maintenance of rodents. Studies have shown that in case of *T. brucei* infections, microscopic blood examination detects only 5% of the proportion of the parasites whereas mouse inoculation picked up 95% of infections [27]. Unlike *T. brucei* infections, 80% of *T. congolense* infections were determined by blood examination.

Immunodiagnostic techniques:

The limitations associated with direct demonstration of parasites led to the development of alternate techniques providing indirect evidence of infection. Although these immunodiagnostic tests do not help in the control of infection, they help in the diagnosis with increased diagnostic sensitivity and specificity. For the detection of trypanosome antibodies in animals and humans, the indirect fluorescent antibody test (IFAT) has been used. The IFAT has been shown to be specific and sensitive to some extent for detecting antibodies in trypanosome infected cattle [29]. The other test used is enzyme-linked immunosorbent assay (ELISA). Latex agglutination card test can be carried out in the field. However this test is not ideal for screening large numbers of animals for epidemiological studies and control programmes. The card agglutination test for trypanosomiasis has been used for the detection of infections [26]. These tests detect antibodies against the parasite, but there are several shortcomings for antibody detection. The most important limitation is the use of ill-defined antigens and since the antibodies may persist for several months following recovery, the serum antigens may not reflect an existing infection. Antigen detection ELISA is a breakthrough in trypanosome diagnosis as the antigen positivity indicates existing infection. The diagnostic strategy for the future is a combination of one or more sensitive trypanosome detection techniques with antigen-trapping ELISA [26].

1.1.9 Treatment

The development of vaccine is difficult due to the ability of trypanosomes to constantly modify their VSG surface coat. Therefore, chemotherapy is currently the best available option so far for controlling infections in humans and animals. The major drugs used for animal trypanosomiasis are homidium chloride, isometamidium chloride and diminazene aceturate [30, 31]. Chemotherapy has several strengths and limitations in the control of animal trypanosomiasis.

Only few drugs are available for treatment of African animal trypanosomiasis and the available drugs are very old and most of them have been in use for more than 50 years. Limited therapeutic efficacy, emergence of drug resistance and serious side effects are the major problems associated with these drugs. The big gap between production price and expected therapeutic outcome is another major concern associated with chemotherapy.

1.1.10 Control

African trypanosomiasis control attempts have a long history and dates back to the period when the European powers were really concerned about the chronic loss of livestock affecting the agriculture and rural development [32]. There is no available vaccine or effective drug available for African trypanosomiasis. The control measures were based on active detection and treatment, which was combined with tsetse control programmes including bush clearance, and insecticidal spraying of tsetse resting sites. In the beginning, these were largely successful. However, the incidence of the disease began to rise by 1960s when the progressive withdrawal of colonial infrastructure affected the routine tsetse control activities. Another control measure involves the release of laboratory reared radiation sterilized male tsetse were successful to some extent [33]. In general, most control measures were mainly focused on minimizing contact with tsetse flies. Among these, vector control measures were cheaper and easier techniques compared to other measures [34]. There was considerable decline in the incidence of sleeping sickness and Nagana from 1920s to 1960s due to the effective control measures.

1.1.11 Mouse models in African trypanosomiasis: Animal models of African trypanosomiasis have been used for many years to investigate the disease pathogenesis and to test novel drug therapies. Although mouse models have their own limitations, they have contributed significantly to the understanding of factors that regulate immunity to African trypanosomiasis.

The inbred mouse models are valuable tools in the research of immunopathogenesis of African trypanosomiasis as they enable genetic studies of particular phenotypic differences [35]. C57BL/6, BALB/c and C3H/He are the most frequently used mouse strains to investigate the resistance/susceptibility to African trypanosomiasis. The C57BL/6 mice is the relatively resistant strain and show longer survival and better parasite control than BALB/c and C3H/He mice [35]. BALB/c and CBA mice are the most susceptible phenotypes following *T. congolense* infections, succumbing to their infections within 10 days [36]. In contrast, *T. congolense*-infected C57BL/6 mice control several waves of parasitemia and survive up to 100 days. C3H, CFLP and A/J mice survive about 25 days after infection [36, 37]. C3H/He, DBA/1, DBA/2 and BALB/c are the most susceptible strains in *T.b.rhodesiense* infections whereas A/J mice is the intermediate strain and C57BL/6 mice is display a relatively resistant phenotype [38].

The use of different knockout mice for *in vivo* studies has provided a new dimension to trypanosome research as antibody mediated neutralization of immune factors is not always satisfactory. *T. congolense* infection is associated with excessive pro-inflammatory cytokine production and over production of other regulatory cytokines. Therefore anti-cytokine antibody treatment and several knock-out mouse models have been used in studying the role of each cytokine and immune factor in immunity to African trypanosomiasis. Indeed, the need for an early inflammatory response in efficient parasite control in *T. congolense* infections were demonstrated and explained by using IFN- γ deficient mice. The role of antibodies against the VSG has been studied in detail using total B cell-deficient mice or IgM or IgG deficient mice [39]. Various immune regulatory and effector molecules are involved in trypanosome control. In *T. congolense* infections TNF deficient mice and inducible nitric oxide synthase deficient

mice (iNOS^{-/-}) have been used to study role of TNF and nitric oxide (NO), respectively, in controlling the infection [40].

1.2 Immune Response to African Trypanosomiasis

1.2.1 Polyclonal B cell activation

Polyclonal B cell activation is a characteristic immunologic feature of African trypanosome infections. There is an increase in immunoglobulin levels especially IgM in sera of infected animals [41]. This increase in serum Ig levels in African trypanosomiasis patients have been shown to be due to polyclonal B lymphocyte activation [42]. However, the precise mechanism triggering the B cells to proliferate and differentiate into antibody-producing cells during the course of trypanosomiasis is still unknown. Polyclonal B lymphocyte activation also leads to the production of antibodies that are not related or specific to trypanosomal antigens resulting in hypergammaglobulinemia [43]. It has been suggested that this nonspecific polyclonal B lymphocyte activation also occur during experimental murine trypanosomiasis. For example, in *T. brucei* infected mice, the number of IgM antibody-producing cells increase in the spleen, and this is accompanied by increased blastogenesis [44]. As a result of polyclonal B lymphocyte activation, some autoantibodies have shown to develop during the course of African trypanosomiasis [44].

The precise cause of polyclonal B cell activation in African trypanosomiasis is not known. It has been shown that African trypanosomes possess B cell mitogens that are able to cause polyclonal activation of B cells. Indeed, the total number of splenic lymphocytes (including B cells) increases 15 times after 3 weeks of infection and these include cells that produce antibodies against a variety of antigens [44]. Mitogenic stimuli derived from parasites may be responsible

for the correlation existing between blood parasitemia and development of polyclonal antibody synthesis.

Binding of VSG to the C3 fragment of the complement receptor on B cells could lead to polyclonal activation. The soluble fragment of *T. congolense* VSG has been shown to form a covalent product with C3b, a breakdown component of C3 in the presence of variant specific antisera [45]. However the mechanisms underlying VSG-C3b complex induced polyclonal B cell activation is not still fully understood.

Polyclonal B cell activation is considered as an evasion mechanism of blood stream forms of African trypanosomes as the insect stages of trypanosomes which lack the VSG, do not induce polyclonal B cell activation [46]. Polyclonal activation prevents selective proliferation and high affinity antibody production against the parasite because the process restricts affinity maturation that is critical for formation of antibodies with high affinities [43, 47]. In addition, there are reports suggesting that polyclonal B lymphocyte activation may be one of the mechanisms of the generalized immunosuppression that occurs in trypanosomiasis [44]. In addition, the prevention of maturation of high affinity antibodies to the parasite antigens due to polyclonal activation provides another evasion mechanism for the parasite.

1.2.2 Immunosuppression

One of the characteristic features of African trypanosomiasis in human, cattle and mice is the profound suppression of the host immune response, which results in enhanced susceptibility to opportunistic infections [48, 49]. This generalized immune suppression of the host has been documented to affect both humoral and cellular immune functions, which eventually leads to the occurrence of trypanosome-induced immunopathology [50, 51]. Greenwood et al showed that the generalized immunosuppression associated with trypanosomiasis in patients was the major

cause of increased susceptibility of those individuals to opportunistic infections [52]. In line with this, *T. congolense* and *T. vivax* infections of cattle leads to suppression of antibody responses to a variety of vaccines [53]. In a similar fashion, sheep infected with *T. congolense* displayed suppressed antibody response to *Brucella abortus* [54].

Studies in laboratory rodent models have demonstrated suppression of both B and T cell responses in trypanosome infections as well. Dysfunctions of T cells results in unresponsiveness to mitogens and alloantigens [55, 56]. However the mechanism by which trypanosomes cause suppression of cell-mediated immunity is not clear. It has been shown that an active ongoing infection is necessary for trypanosome-associated immunosuppression [55]. Treatment of *T. congolense* infected mice with Berenil has shown to abolish the infection-associated immunosuppression [57]. In *T. congolense* infected rabbits, both *in vivo* and *in vitro* correlates of cell-mediated immunity have shown to be depressed [55]. Although the trypanosome infection-associated immune response is known for a long time, the mechanism behind it or the cell types responsible for this suppression is not very well known.

African trypanosome infections are associated with increased IgM levels and the expansion of B cell populations [58]. Most of the IgM antibodies have shown heterophilic and autoimmune specificities and it has been postulated that trypanosomes possess a mitogen able to non-specifically activate the B cells. It is speculated that polyclonal B lymphocyte activation could be one of the mechanisms responsible for the suppressed immune response of *T. congolense* infected mice. Polyclonal activation could results in the exhaustion of antigen specific B lymphocytes and may cause immunosuppression [44]. Therefore polyclonal activation and immunosuppression are co-existing mechanisms in trypanosome infections with devastating consequences. Macrophages with suppressive phenotype [59], suppressor T cells [48, 60],

various inflammatory mediators including nitric oxide [61] and cytokines have all shown to play a role in trypanosome-induced immunosuppression.

1.2.3 Role of Nitric Oxide:

Nitric oxide is an effector molecule involved in the regulation of immune system and are released by macrophages and other cells after immunological activation [62]. The enzyme, nitric oxide synthase, is involved in the synthesis of nitric oxide (NO) from the amino acid L-arginine. The parasiticidal activities of macrophages have been attributed in part to their inducible nitric oxide synthase (iNOS) gene expression and NO production [63, 64]. This is related to the levels of IFN- γ production by T cells. Macrophages of trypanosoma-infected hosts are sources of many pro-inflammatory cytokines and inflammatory mediators like IL-1, IL-6, IL-12, TNF and NO [20, 65]. NO is the most pivotal effector molecule that has both cytostatic and cytotoxic properties for the parasite [66]. Our group and others have shown that following infection of macrophages with *T. congolense*, there is NO production in macrophages and this associated with a coordinated activation of MAPK, STAT1 and GAS elements for the effective expression of iNOS/NO [67-69]. In experimental *T. brucei* infection, by using NO synthase (iNOS^{-/-}) deficient mice, it has been reported that NO is not involved in parasitemia control [70]. However, it has been shown that control of *T. congolense* parasitemia is severely impaired in the absence of NO production and the NO production is dependent on IFN- γ R signaling [71]. By using iNOS deficient mice, Magesz *et al.* showed that inducible nitric oxide synthase (iNOS) dependent trypanosome killing occurs downstream of IFN- γ -R signaling, suggesting that the host protective immunity is partly dependent on NO [71].

In experimental *T. brucei* infections in mice, NO produced by macrophages is a mediator of immunosuppression [61]. The stimulation of macrophages by IFN- γ in synergy with TNF- α

induces the synthesis of high amounts of NO, which is associated with immunosuppression [60, 61]. In *T. congolense*-infected mice, NO do not mediate suppression of T cell proliferative responses [50]. Interestingly, in *T. congolense*-infected cattle, NO production by macrophages and monocytes are depressed and NO does not mediate T cell suppression in this model [72]. The NO synthase mRNA was also depressed in macrophages of the parasite infected cattle [72]. Therefore suppression of T cell responses by NO in trypanosomiasis may depend on the strain and the parasite species as well as the host.

1.2.4 Role of pro-inflammatory cytokines:

The precise role of individual cytokines has not been studied in detail in African trypanosomiasis. However profound dysregulation of cytokine network is a hallmark feature of the disease and it is well documented that cytokine responses influence the outcome of African trypanosomiasis [73, 74]. The effect of cytokine in the course of trypanosomiasis is dependent on the timing, microenvironment and dosage. Type 1 cytokines have been shown to confer resistance to African trypanosome infections by limiting parasite growth during the early stage of infection [73, 74]. However sustained Type 1 cytokine response may be harmful and promote disease progression [75, 76]. Because of the pleiotropic nature of cytokines, the interpretations of results of experiments with cytokines are difficult in African trypanosomiasis. It has been shown that anti- IFN- γ treatment prevents the early death of highly susceptible *T. congolense*-infected BALB/c mice. Anti-IFN- γ treatment resulted in very low parasitemia, control of several waves of parasitemia and over four fold increase in survival period in the highly susceptible BALB/c mice [77]. However, infection of IFN- γ -knockout mice showed that IFN- γ is required for the survival of relatively resistant *T. congolense* or *T. brucei* infected C57BL/6 as these mice failed to control their first wave of parasitemia and succumbed acutely to the infection [71, 73].

IFN- γ is crucial in the activation of macrophages by inducing the synthesis of various cytokines like TNF, IL-12 and in the production of NO. IL-10 has been shown to be critical for trypanotolerance. Infection studies using IL-10^{-/-} mice showed that deficiency of IL-10 resulted in a striking reduction in the survival time in *T. congolense*- and *T. brucei*-infected mice and this was associated with increased levels of serum pro-inflammatory cytokines like IFN- γ , TNF and NO [39, 78].

1.2.5 Role of Complement:

Persistent hypocomplementemia is a profound feature of African trypanosomiasis and several studies in cattle, sheep and mice have suggested that the complement system might be involved in the battle between the host defense and the parasite [54, 79, 80]. Previous reports suggest that both the alternative and the classical pathways of complement are activated during trypanosomiasis [81, 82]. Although the efficiency of parasite clearance and immune complex removal is enhanced by complement, clearance of trypanosomes can occur by phagocytic cells even in the absence of complement [83, 84]. During the early stage of infection, activation of the alternative pathway that occurs in the absence of specific humoral antibodies plays a crucial role in parasite clearance [85]. The classical pathway, mediated by trypanosome specific antibodies help in parasite clearance by antibody mediated lysis or opsonization [86]. A study that investigated the levels of proteins of the alternative pathway of the complement system in different mice strains infected with *T. congolense* observed that the amount of Factor B in the plasma of infected mice during the later stages of infection was positively correlated with survival time and was higher in the relatively resistant C57BL/6 mice than in the susceptible BALB/c mice [80]. A similar study was performed by Ogunremi et al who demonstrated that the deposition of C3b, an important breakdown product of activated alternative pathway of

complement, was higher in C57BL/6 compared to Balb/c mice after *T. congolense* infection [87]. Studies have also demonstrated the activation of the classical pathway of complement following infection with African trypanosomes in animals. Anti-*T. congolense* VSG antibodies can activate the complement system of cattle and mice both *in vitro* and *in vivo* [88, 89]. Interestingly, trypanosomes also have shown to induce depletion through direct cleavage of complement components in the absence of Abs, which could be for their own survival [85, 90]. For example, in cattle infected with *T. congolense* and *T. vivax*, reduction in the serum levels of both total hemolytic component of the alternative pathway and C3 of the classical pathway has been reported [91].

1.2.6 Role of granulocytes:

The role of granulocytes in the outcome and pathogenesis of African trypanosomiasis is poorly investigated. However there are some reports that analyzed changes in the peripheral blood leucocyte populations during tsetse fly transmitted *T. congolense* infection in trypanotolerant N'Dama and trypanosusceptible Boran cattle [92, 93]. The changes in peripheral blood leukocyte populations were examined by using routine hematological procedures in *T. congolense* infected cattle [92]. The studies found no significant changes in total neutrophils from preinfection levels to first peak of parasitemia. Neutrophils or eosinophils in either N'Dama or Boran cattle were not significantly different during the course of infection. However the calves infected with *T. congolense* had very low total white cell count and neutropenia and showed marked reduction in granulocyte mobilization during the first 14 weeks of infection [92]. Although the underlying mechanism for the reduction of different cell population is not known, it has been shown that erythrocytes and neutrophils are destroyed by the cells of mononuclear phagocytic system in *T. congolense* infected cattle. A recent report document that

macrophage migrating inhibitory factor (MIF) is an important pathogenic molecule and mediates pathogenic inflammatory immune response and increases the recruitment of inflammatory monocytes and neutrophils to contribute to liver injury in *T. brucei* infected mice [94]. Interestingly this study also showed that neutrophil derived MIF contributed to the increased pathogenic liver TNF production and liver injury during trypanosome infection [94]. Furthermore, this report shows that the serum myeloperoxidase level, which is an indicator of neutrophil activity, is increased in *T. brucei* infected WT mice than MIF^{-/-} mice. The major cause of tissue pathogenicity in *T. brucei* infected mice could be due to the enhanced hepatocyte death caused by the neutrophils, resulting in the release of necrotic products into the circulation [94].

1.2.7. Role of T cells:

It has been shown that BALB/c mice are highly susceptible to *T. congolense* and *T. brucei* infections where as C57BL/6 mice are relatively resistant. The early mortality of BALB/c mice is associated with enhanced IFN- γ and these mice die due to systemic inflammatory response syndrome [95, 96]. The early mortality of *T. congolense* infected BALB/c mice are prevented by administration of anti IFN- γ antibodies [77]. Blocking the IL-10R in *T. congolense* infected relatively resistant C57BL/6 mice results in elevated IFN- γ levels and leads to early death [95]. The early death in anti-IL-10 R treated mice can be reversed by administration of anti IFN- γ antibodies [95]. Therefore it is concluded that the excessive IFN- γ is leading to the early mortality of these mice. However it has convincingly shown that IFN- γ is required for the survival of relatively resistant C57BL/6 mice by using IFN- γ knockout mice in *T. congolense* and *T. brucei* infections. So an optimal cytokine response is needed at the initial stage of the infection for parasite clearance. Therefore IFN- γ producing T cells have an important role in

African Trypanosomiasis. *T. congolense* infected CD4^{+/+} and CD4^{-/-} BALB/c mice have similar parasitemia and survival time where as infected BALB/c mice partially depleted of CD4⁺ T cells have lower parasitemia and survive longer than the infected normal BALB/c mice [39]. This partial depletion of CD4⁺T cells reduced IFN- γ production without major effect in the production of IL-10 and parasite specific IgG2 antibodies [39]. This further suggests that the early mortality of infected BALB/c mice is due to the excessive IFN- γ production and the IFN- γ in turn exerts pathology by macrophage hyper-activation resulting in excessive cytokine production.

During the early stage of infection, the production of cytokines and expression of co-stimulatory molecules on macrophages favor the development of Th1 responses and suppress Th2 responses [97, 98]. The polarization of Th1 cell responses occurs early in infection, and it is a characteristic feature of African trypanosomiasis. The induction of Th1 cell response and IFN- γ production in infected animals contributes to the overall host resistance to the infection. This early Th1 polarization is associated with upregulation of costimulatory molecules on macrophages that include MHCII, CD80 and CD86 expression.

Macrophages

1.2.5 Macrophages origin and development:

Metchnikoff identified macrophages in the late 19th century by virtue of their phagocytic activity. Further studies on morphology, surface marker and gene expression profile revealed significant differences between the macrophages present at different locations and such phenotypic differences were accompanied by functional diversity [99] [100]. Macrophages are important players of the innate immune system and play critical role in the development and maintenance of homeostasis of the tissues in which they reside [99, 101]. There has been

considerable revision of our understanding of monocyte and macrophage biology. The dogma that tissue resident macrophages are derived from local differentiation of circulating monocytes has been changed and recent studies provide evidence for the existence of monocyte-independent tissue resident macrophages [102, 103]. This finding is a really paradigm shift in the development and origin of macrophages.

The *in vitro* differentiation of monocytes into macrophages was studied in early 1960s by Van Furth group and established the mononuclear phagocyte system (MPS) concept (84, [104]). According to this concept, the homeostasis of tissue-resident macrophages relies on the constant recruitment of blood monocytes. So monocytes and macrophages were considered as two related cell types and there is a developmental continuum between the two. Although under certain conditions, especially during inflammation, monocytes can give rise to macrophages, recent studies questioned the applicability of this dogma. According to the new findings, it can be concluded that monocytes do not contribute to the development of tissue macrophages. Furthermore, the adult tissue macrophages are derived from embryonic precursors and these tissue macrophages can maintain themselves in adults by self-renewal [103]. Thus, adult tissue macrophages are derived from embryonic progenitors that gave rise to fetal tissue macrophages at a very early stage of development [103]. Studies in mice and rat have shown that primitive macrophages appear in the yolk sac blood island of the developing embryo and they undergo a fast-track differentiation pathway to become mature macrophages in fetal tissues [105, 106]. These macrophages resemble the adult monocytes in the expression of CD11b, F4/80, Ly6C and CSFIR [107]. After the emergence of yolk-sac derived macrophages, monocytes are generated in the fetal liver and is differentiated through pro-monocyte intermediates [108].

1.2.6 Macrophage activation:

Macrophages possess remarkable plasticity, which allow them to respond to environmental signals and change their phenotype. Macrophages are key modulator and effector cells in immune response and their activation influences both innate and adaptive immune responses. Macrophage activation is a term used to describe macrophages that have been primed or stimulated to show enhanced effector activities. Depending on the nature of the activating stimuli and the eventual phenotype displayed, activated macrophages are classified into 2 groups classically activated macrophages or M1 type and alternatively activated macrophages or M2 type. The term macrophage activation or classical activation was introduced by Mackaness in 1960s to describe the non-specific, microbicidal activity of macrophages towards *Listeria monocytogenes* [109]. The classical activation denotes the effector macrophages that are produced during cell-mediated immune responses. Classically activated macrophages secreted high levels of pro-inflammatory cytokines and mediators and they exhibited enhanced microbicidal and tumoricidal capacity. IFN- γ , produced by innate or adaptive immune cells, is the major molecule involved in the classical activation of macrophages [110]. Classically activated macrophages are very important in host defense and the cytokines and mediators they produce can lead to host-tissue damage, hence their activation must be tightly controlled. For example in rheumatoid arthritis and inflammatory bowel disease, classically activated macrophages are the key mediators of immunopathology [111, 112].

Macrophages activated in the presence of Th2 cytokines, like IL-4 and IL-13, are called alternatively activated macrophages (M2). *In vitro* treatment of macrophages with IL-4 and IL-13 (i.e. alternative activation) results in the failure of antigen presentation to T cells and therefore minimal cytokines are produced from these macrophages. Alternatively activated macrophages are also less efficient in producing toxic oxygen and nitrogen radicals and in

killing intracellular pathogens than classically activated macrophages [113]. The most important function of alternatively activated macrophages is related to wound healing. There are also evidence for the contribution of alternatively activated macrophages in clearance of helminths and nematodes [114] [115]. For example, in infection with the gastrointestinal helminth parasite *Heligmosomoides polygyrus*, alternatively activated macrophages function as important effector cells and contribute to the parasite clearance [114].

Similar to M1 and M2 macrophages, another set of macrophages called regulatory macrophages have been demonstrated to arise following certain innate or adaptive immune responses, particularly associated with stress [116]. Stress leads to release of glucocorticoids by adrenal cells resulting in the inhibition of macrophage-mediated host defense and inflammatory functions due to inhibition of pro-inflammatory gene transcription [117]. The primary function of regulatory macrophages is to dampen the immune response and limit inflammation. Hence, regulatory macrophages usually arise at the later stages of adaptive immune response [118]. Although macrophages exhibit plasticity, it is not as well defined as that of T cells and reliance on a single biochemical marker to identify a particular macrophage population is difficult [118]. However, some studies have shown that the phenotypic switch occurs in the macrophage population over time [118].

1.2.7 Macrophage surface receptors and antigens:

The surface receptors of macrophages regulate a number of functions including cell differentiation, growth and survival, migration, phagocytosis, activation and cytotoxicity. The ability of macrophages to recognize a wide variety of endogenous and exogenous ligands is central to their role in homeostasis as well as host defense in innate and acquired immunity, inflammation and immunopathology.

1.2.7.1 Immunoglobulin Fc receptors:

Cell surface receptors for the Fc portion of the immunoglobulins (FcR) are important for various functions of macrophages such as endocytosis, antibody-dependent cellular cytotoxicity and release of potent inflammatory agents. There are 3 distinct classes of Fc receptors on macrophages: Fc γ RI, Fc γ RII, and Fc γ RIII. The FcRs are further divided into activating and inhibitory receptors based on the presence or absence of tyrosine-based activation motif (ITAM) in their intracytoplasmic domains [119]. The activating receptors are Fc γ RI, Fc γ RII and Fc γ RIII. The inhibitory Fc γ R IIB has an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain [119]. Fc γ RI can bind to monomeric IgG with high affinity and is therefore referred to as the high affinity receptor. In the presence of IFN- γ , the expression of Fc γ RI on macrophages is increased [120]. The major functions of Fc γ RI are antibody-dependent cellular cytotoxicity, phagocytosis and cytokine synthesis [120]. The function of all Fc γ Rs expressed on the cell surface is the internalization of opsonized material or immune complexes and it is independent of whether they have ITAM or ITIM. Although all Fc γ Rs have phagocytic function, the molecular mechanisms underlying the internalization are different. In addition to capture and internalization of immune complexes, most Fc γ Rs induce ITAM or ITIM mediated intracellular signaling, which influences the core functions of macrophages. The balance between the triggering of ITAM bearing Fc γ Rs and ITIM bearing Fc γ RIIB determines the nature, quality and outcome of immune complex-mediated activation of macrophages. It has been shown that macrophages from Fc γ RIIB $^{-/-}$ mice have lower activation threshold than WT macrophages and Fc γ RIIB $^{-/-}$ mice are more sensitive to immune complex-induced arthritis and sepsis [121, 122]. Several studies suggest that the increased activation that occurs in the absence of Fc γ RIIB

signaling increases the ability of macrophages to clear bacteria and can be beneficial for the host, but at the same time it can be detrimental as it increases immunopathology [123].

1.3.3.2 Complement receptors

Phagocytosis is the primary function of macrophages and is facilitated through opsonization, which is a process of tagging pathogens for recognition by serum components. Complement-mediated opsonization triggers all the three pathways of complement activation including the classical, lectin and alternate pathway [124]. Although the initial biochemical steps leading to the activation of the central complement component C3 is different in these pathways, they share the common step of activating this central component. The pivotal step in opsonization in all three pathways is the conversion of the component C3 to C3b. Several studies have shown that macrophages have 3 distinct complement receptors: CR1, CR3 and CR4 [125, 126] [127]. It has been shown that CR3 and CR4 play a role in the clearance of opsonized apoptotic cell by human macrophages. CR1 (CD35) is the receptor for C3b and C4b and participates in the engulfment of opsonized particles coated with C3b and C4b [128]. CR1 has a main role in microbial recognition and act as a cooperative receptor that facilitates both Fc γ R and CR3-mediated endocytosis [129]. CR3 is a member of β_2 integrin family of adhesion molecules and is expressed on a variety of cells including mononuclear phagocytes, neutrophils and NK cells.

1.3.3.3 Mannose receptors

Mannose receptor (MR) is a carbohydrate binding receptor present on macrophages, dendritic cells and non-vascular endothelial cells. MR (CD206) is a type 1-membrane molecule with three types of extracellular domains, which recognizes endogenous and exogenous ligands. Therefore MR has important roles in homeostatic process and pathogen recognition. It is an endocytic receptor which constantly recycles between plasma membrane and the early endosomal

compartment even in the absence of interaction with a ligand [130]. About 10-30% of the receptor is found on the cell surface at the steady state and the remaining 30% has been shown to localize internally [130]. The major functions of MRs are clearance of endogenous molecules, promotion of antigen presentation and modulation of cellular activation. However, it has been demonstrated that MR has a role in the phagocytosis of various pathogens such as *Mycobacterium tuberculosis*, *Francisella tularensis* and *Candida albicans* [131-133]. Thus, MRs act as a bridge between homeostasis and immunity and is considered as a non-canonical pattern recognition receptor, which is able to bind endogenous molecules as well as pathogens, and mediates physiological clearance. In addition, recent studies suggest that MR participates in intracellular signaling leading to target gene expression [134, 135]. Because MRs lack signaling motifs at the cytoplasmic region, they acquire assistance from other receptors to trigger the signaling cascade. Similarly, because of its role in antigen processing, they have been shown to provide a link between innate and adaptive immunity [136]. Ligation of macrophage mannose receptors by particles or microorganisms triggers various responses leading to the release of inflammatory mediators, cytokine production and modulation of other cell surface receptors [125, 136]. The role of mannose receptors in African Trypanosomiasis is not yet studied.

1.3.3.4 Scavenger receptors

Scavenger receptors are structurally heterogeneous receptors expressed by macrophages, which can identify and remove unwanted entities by the recognition of modified self-molecules like apoptotic cells or by recognition of non-self molecules like microorganisms. They were originally identified by their ability to remove modified lipoproteins but now it is known that they can take part in a variety of functions such as pathogen clearance and lipid transport. Individual scavenger receptor has the ability to bind to a wide range of co-receptors, which

make their responsiveness highly versatile. SR-AI, SR-AII, MARCO, CD163, CD36 and LOX-1 are the selected scavenger and functionally related receptors expressed by macrophages [137]. They have been implicated in the uptake of polyanionic ligands and in the uptake of apoptotic cells.

SR-A is well expressed at the surface of macrophages and a large part of SR-A is expressed intracellularly within the endocytic compartment [137]. Studies using *Staphylococcus aureus* and *Listeria monocytogenes* have shown that SR-A has a role in microbial resistance and susceptibility to bacterial infection *in vivo* [138]. MARCO is a macrophage restricted SR-A family member. Although it resembles to SR-A, MARCO is a product of a distinct gene and the expression pattern is different. MARCO binds to a wide range of gram positive and gram-negative bacteria and contributes to the localization and clearance of circulating organisms [139]. CD36 is very different from SR-A in structure and is a double spanning SR. It is expressed on macrophages and certain other cell types and is known as a SR for oxidized LDL and apoptotic cells [137]. Although the role of SR in African trypanosomiasis is not known, it has been demonstrated that, a member of the SR family has a role in the recognition and binding of sVSG to the cell surface and the SR recognizes a component other than mannose structures present on GPI core glycan [140]. CD36 has been implicated in the recognition of apoptotic cells.

1.3.3.5 Cytokine receptors

Macrophages display a number of cytokine and chemokine receptors and these receptors have an important role in the generation, differentiation, migration and activation of these cells. These include the receptors for IFN, TNF, IL-1, IL-1 IL-6, IL-13, GM-CSF, M-CSF and a number of chemokines [141].

1.3.3.6 Triggering receptors expressed by myeloid cells (TREMs)

TREM family of molecules consists of four myeloid transmembrane glycoprotein receptors and TREM1, TREM2 and TREM3 are well characterized. TREM1 is expressed by macrophages and neutrophils and has an important role in inflammation as it induces the expression of IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 α (MIP-1 α) [142]. TREM1 has a potential role in amplifying the inflammation. Blockade of TREM1 *in vivo* has shown to prevent shock and death in septic shock models with *E. coli* or cecal ligation and puncture [143]. TREM2 has shown to bind to anionic carbohydrates on bacteria and yeast and thus they have scavenger receptor like properties and therefore may play a role in pathogen recognition [144].

1.2.8 Functions of macrophages

1.3.4.1 Macrophages in inflammatory diseases

Following an infection or injury, differentiated macrophages migrate to the affected tissues. The recruited macrophages show a pro-inflammatory phenotype and secrete a variety of inflammatory mediators including TNF, IL-1 and nitric oxide, which collectively activate anti-microbial defense mechanisms and contribute to the killing of invading organisms [145]. IL-12 and IL-23 produced by these macrophages direct the differentiation and expansion of Th1 and Th17 cells and help to drive the inflammatory response forward [146]. As these inflammatory macrophages facilitate the clearance of invading organisms, they are beneficial at the early stage but collateral tissue damage is triggered because of the toxic activity of reactive oxygen and nitrogen species. In order to counteract the tissue damaging effect of inflammatory macrophages, they either undergo apoptosis or switch to an anti-inflammatory or suppressive phenotype, which dampens the pro-inflammatory response. Several studies have shown that

macrophages have important roles in many chronic inflammatory diseases like atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, and fibrosis [147, 148]. The contribution of macrophages to these diseases varies in different stages of the disease and is controlled by many factors. In a variety of autoimmune diseases, many key inflammatory cytokines are produced by macrophages including IL-12, IL-18, IL-23 and TNF, which are identified as drivers of autoimmune inflammation [149]. It has been shown that end stage joint autoimmune inflammation is promoted by macrophage-derived IL-23 [150]. Macrophages and dendritic cells that produce IL-18 and IL-12 have been shown to be responsible for IFN- γ and TNF-dependent chronic polyarthritis in mice. Similarly, macrophages and dendritic cells are shown to be the key producers of many cytokines that are implicated in the pathogenesis of inflammatory bowel disease.

1.3.4.2 Macrophages in fibrosis

As a part of the normal homeostatic function in tissues, macrophages phagocytose and clear apoptotic cells. However when they encounter invading organisms or necrotic debris after injury, they get activated by endogenous danger signals and pathogen associated molecular patterns. Upon activation, they produce anti-microbial mediators that help to kill invading pathogens and thus assist in the restoration of tissue homeostasis. But activated macrophages are also responsible for the production of a variety of inflammatory cytokines and chemokines, which exacerbates tissue injury followed by aberrant wound healing, and if this response is not adequately controlled leads to fibrosis. Researchers have focused recently on elucidating the mechanisms that suppress inflammation and prevent fibrosis. Tissue damage could be kept to a minimum if the dominant macrophage population converts from pro-inflammatory phenotype to anti-inflammatory, suppressive or regulatory phenotype. It has been shown that in addition to

promoting fibrosis, macrophages are involved in the recovery phase of fibrosis by dampening the immune response that contributes to tissue injury and by phagocytizing apoptotic myofibroblasts [151].

1.3.5. Secretions by macrophages

Macrophages secrete several biologically active substances including proteins (enzymes), lipids, nucleotide and oxygen metabolites. The enzymes participate in a wide range of functions like accelerating inflammation, clearing up the debris in inflammation sites, killing tumors etc. Some of the important enzymes include lysozyme, arginase, coagulation factors, lipoprotein lipase etc [152]. The inflammatory and activated macrophages secrete several neutral proteinases like collagenases, elastase and plasminogen activators [153-155]. Macrophage elastase is a metalloproteinase and contributes about 0.1% of the total protein secreted by thioglycolate stimulated mouse macrophages [156]. Macrophages also secrete angiotensin converting enzyme, which cleaves angiotensin I to form an active vasoconstrictor angiotensin II [157]. Other enzymes produced by macrophages include several non-proteolytic enzymes like arginase, lysozyme and lipoprotein lipase [158, 159]. Some enzyme inhibitors like α 2- macroglobulin and plasminogen activator inhibitor are also released by macrophages [160]. In addition, macrophages are major producers of plasma proteins (including fibronectin, apolipoprotein E, coagulation factors and complement components) that are known to participate in inflammation, tissue repair, immunoregulation and molecular transport [161, 162]. Macrophages also secrete some molecular weight substances like reactive metabolites of oxygen and bioactive derivatives of arachidonic acids. These reactive metabolites of oxygen participate in extracellular killing of microbes and tumors while arachidonic acid derivatives participate in inflammation, hypersensitivity and immunoregulation. The wide variety of biochemical substances produced

by macrophages play important role in regulating the physiological and pathologic functions of these cells.

1.3.6. Cytokine production by macrophages

Macrophages are a major source of different cytokines involved in immune response, hematopoiesis, inflammation and other homeostatic process. In response to microorganisms, danger signals or endogenous factors like cytokines, macrophages can de novo synthesize and release various cytokines and chemokines such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF, IFN, MCP-1, MIF, M-CSF, GM-CSF, MIP, TGF beta [163]. These cytokines modulate macrophage function and cell surface marker expression and results in the activation of innate immune system [163] [164]. Macrophages are found at different locations and their capacity to produce cytokines depends upon their localization and maturation [163] . Certain cytokines produced by some other cell types can influence (upregulate or down-regulate) cytokine synthesis by macrophages. For example, T-cells products like IL-4, IL-10, IL-13 results in down-regulating the cytokine synthesis by macrophages. In contrast, IFN- γ and TNF produced by certain activated T cells, upregulate cytokine production in macrophages. In addition to this, macrophages themselves are also important source of inhibitory cytokines including IL-10 and TGF- β [165]. Therefore it is clear that the contribution of macrophages in inflammatory process is limited by IL-4, IL-10, IL-13 and TGF- β , cytokines that reduce the synthesis of pro-inflammatory cytokines. Inflammatory cytokines recruit other immune cells and contribute in determining the fate of other cells. The pro-inflammatory cytokines produced by macrophages are normally protective; however under certain conditions, these cytokines could be detrimental causing chronic disease.

Another molecule that strongly activates macrophages leading to the synthesis and secretion of an array of cytokines is LPS. Following LPS stimulation, TNF is produced very early and IL-6 is secreted in an overlapping time frame [166]. This inflammatory cytokine release is followed some time later by secretion of anti-inflammatory mediators like IL-10, IL-4 and TGF- β to shut off the pro-inflammatory cytokines and thus limit the inflammatory response [167]. The early released TNF by macrophages results in the activation and recruitment of other cells to the site of microbial invasion and infection. However, excessive secretion of TNF causes a significant pathological problem [168].

1.3.7 Role of macrophages in African trypanosomiasis

Macrophages are professional antigen presenting cells and are the first line of defense against pathogens. They help in phagocytosis and release pro-inflammatory cytokines and other mediators [63, 112]. Macrophages are able to recognize phagocytic targets and ingest them at a higher rate similar to neutrophils and monocytes. They also have the ability to modulate their phagocytic functions in response to activation and differentiation signals.

Macrophages play a crucial role in the control of many protozoan parasitic infections including African trypanosomiasis. Intact monocytic cell system is important for the initiation and maintenance of anti-trypanosome responses [169]. Mononuclear phagocytic system has a crucial role in the phagocytosis of opsonized trypanosomes, which is the major mechanism for removal of trypanosomes from blood stream. The involvement of macrophages in experimental African trypanosomiasis has been demonstrated in many studies. For example, during the course of trypanosome infection, the numbers of macrophages are greatly increased in many organs including the liver, spleen and lymph nodes and these cells display morphological and functional features of activation [170, 171]. Because of their role in phagocytosis and production of pro-

inflammatory cytokines, macrophages are vital for both clearance of *T. congolense* and in mediating immunopathology in infected mice. Macrophages in general and Kupffer cells in particular play a key role in trypanosome clearance and in mediating systemic and local responses by releasing pro-inflammatory cytokines [95]. The activation of macrophages during trypanosome infection is due in part to their exposure to parasite components and host-derived IFN- γ , which is produced in response to parasite antigens [172]. These parasite antigens include invariant membrane, cytoplasmic, nuclear antigens and the variant surface glycoprotein (VSG) molecules. Although the exact mechanism of attachment and phagocytosis of trypanosomes by macrophages is not fully understood, these processes have been shown to occur in the presence of variant-specific antibodies [173, 174].

Macrophage activation following infection with different species of African trypanosomes has been studied. In experimental *T. brucei* infection, a large percentage of cells in the enlarged spleen exhibit membrane and functional characteristics associated with activated macrophages. These activated macrophages release enhanced IL-12, TNF and iNOS within the first 2 weeks of infection and this is associated with modulation of host immunity and resistance [60]. In *T. congolense* infection, complement and antibody-mediated phagocytosis by splenic and liver (kupffer cells) macrophages is one of the primary mechanism by which trypanosomes are cleared from an infected host [86, 175]. The parasites coated with antibodies including IgM and IgG are taken up by macrophages, and this results their activation and production of pro-inflammatory cytokines[81]. In addition, activated macrophages present the trypanosomal antigens to T cells in an MHC class II-dependent manner resulting in the production of IFNs. This leads to further activation of macrophages leading to enhanced cytokine production. During the peak parasitemia, there is massive phagocytosis of trypanosomes leading to hyper-activation

of macrophages and increased production of monokines and T-cell cytokine IFN- γ . This systemic cytokine over production leads to systemic inflammatory response like syndrome and results in the death of trypanosome-infected mice.

1.3.8 Role of adaptor proteins in African Trypanosomiasis

The TLR-mediated intracellular signaling events are initiated by binding of different TLRs with their corresponding ligands resulting in the recruitment of a number of adaptor proteins. Myeloid differentiation primary response protein 88 (MyD88) is the major adaptor protein that is a part of almost all TLR signaling pathways. The other adaptor proteins are TIR-domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM) [176, 177]. The activation of these adaptor molecules following ligation of TLRs with their specific ligands leads to the activation of various transcription factors like nuclear factor NF κ B, and activating protein-1 (AP-1) resulting in initiation of specific immune response. The role of MyD88 in host resistance and pathogenesis of parasitic diseases have been explored by infecting MyD88 deficient mice. Infection of MyD88 deficient mice with *T. cruzi* and *T. brucei* results in increased susceptibility due to impaired IL-12 and IFN- γ production [98, 178]. In these experimental models, it was proposed that signaling through MyD88 in innate immune cells has a protective role by activating Th1 response [98, 178]. In contrast, in some experimental models, the decreased pro-inflammatory responses resulting from the lack of MyD88 signaling is beneficial to the host. There is currently no report on the role of MyD88 in *T. congolense*-induced signaling and cytokine production and resistance to the infection. In the third part of this thesis, I used BMDM from MyD88 $^{-/-}$ mice to investigate the role of MyD88 in *T. congolense*-induced MAPK and STAT phosphorylation and production of proinflammatory cytokines by *T. congolense*-infected macrophages.

1.3.9 Signaling pathways for cytokine production

The major signaling pathways that are known to induce cytokine production in immune cells include mitogen activated protein kinase pathway (MAPK), JAK-STAT pathway and NFκB pathway. MAPK and STATs are cytoplasmic proteins that play critical role in immune regulation, cytokine production and inflammatory response. Studies show that MAPK and STAT family members coordinate and propagate multiple inflammatory immune responses [179, 180]. The activation of MAPK, STAT and NFκB pathways initiate a cascade of intracellular events culminating in the expression of various pro-inflammatory genes.

1.3.9.1 MAPK pathway

MAPKs are a highly conserved group of serine/threonine protein kinases that mediate intracellular signaling events necessary for carrying out a variety of fundamental cellular processes such as proliferation, differentiation, motility, stress response, apoptosis, and survival. Three major families of MAPKs are recognized including extracellular signal-regulated kinases (ERK), p38MAPKs and c-Jun N-terminal kinase/stress-activated protein kinases (JNK-SAPKs) [181, 182]. A broad range of extracellular stimuli including mitogens, cytokines, growth factors, bacterial products and environmental stressors, has been shown to activate the MAPK pathway in macrophages. The signals transmitted through different cascades modulate the activity of numerous transcription factors and other regulatory proteins to ensure proper cellular responses. Localization of individual proteins, transcription of genes and increase cell cycle entry are some of the functions mediated by MAPK signaling. ERK is an essential gene and animals that lack ERK die during embryonic development [183]. ERK has different isoforms from ERK1-ERK8, but ERK 1 and ERK2 are most important among them. Reports show that germ-line mutations in ERK1/2 cascades are associated with developmental abnormalities like cardio-facio-cutaneous

syndrome [184]. In addition, aberrant functions of MAPK has reported in several diseases like cancer, diabetes and obesity [185]. Activation of MAPKs results in specific cellular response by phosphorylation of a wide range of substrates including transcription factors and cytoskeletal proteins and MAPK pathways are finely regulated by various mechanisms including the scaffolding of MAPK cascades and phosphorylation and dephosphorylation of MAPKs [186]. The addition of a phosphate group (phosphorylation) causes conformational change in the phosphorylated protein, which ultimately leads to the activation or inhibition. The addition of phosphate group is catalyzed by protein kinases. In addition to the activation or inactivation, phosphorylation can also modulate the action of an enzyme or change the specificity of the enzyme. A variety of cellular functions are regulated by MAPKs, which is mediated by relaying the extracellular signals to intracellular response. MAPK signaling cascade plays a major role in the signaling of a wide variety of extracellular agents, which is operated through various receptors. Upon stimulation, ERK1/2 phosphorylates different substrates in many cellular locations resulting in the induction of ERK1/2 dependent cellular processes like proliferation, differentiation etc. [187].

Four isoforms of p38 MAPK (p38 α , β , γ , δ) have been identified. P38 α is the most predominant form in monocytes and in macrophages p38 α and p38 γ are abundant while p38 β is absent. Even though some reports suggest that p38 α is the most important isoform in inflammation, there is clear evidence that all four p38 isoform activation may play a vital role in inflammation [188]. P38 MAPK pathway has a major role in regulating the pro-inflammatory cytokine biosynthesis at the transcriptional and translational levels therefore the components of this pathway has gained importance as therapeutic targets of inflammation related diseases[189] [188]. P38 cascade is mainly activated by stress-related stimuli and they have also shown to

respond to signals transmitted by a wide variety of other receptors and cellular processes [190, 191]. P38 also plays a critical role in the regulation of immunological effects, apoptotic, cellular senescence and even survival and this suggests that dysregulation of the cascade results in several pathological manifestations [192, 193]. P38 has also shown to induce diabetes, cancer and neurodegenerative diseases [194, 195].

JNK cascade is also known as stress activated protein kinase cascade (SAPK) and it has shown to be stimulated by stress or stress independent stimuli and other receptors including mitogens. JNK phosphorylate a large number of substrates both in the nucleus and in the cytoplasm upon stimulation. These phosphorylated targets further regulate the transcription of many genes mediating different cellular processes like apoptosis, immunological effects and neuronal activity and dysregulation of JNK cascade leads to several diseases [196, 197].

1.3.9.2 STAT pathway

STATs were first described by Darnell *et al* [198] as transcription factors induced by ligands in IFN-treated cells. Later several other groups showed the critical role of STATs in signal transduction pathways by cytokines and growth factors [199]. Seven mammalian STAT-encoding genes have been identified till date. All STATs have 7 well-defined domains including an N-terminal conserved domain and a C-terminal transactivation domain. The amino-terminal region prevents dimerization of STATs in their inactive state [200]. SH2 domain is critical for the recruitment of STATs to activate receptor complexes and also for the interaction with Janus (JAK) and Src kinases. It is the most conserved domain among STATs and plays an important role in STAT signaling by facilitating homodimerization and heterodimerization, which are crucial for nuclear localization and DNA binding activities [201]. In an inactive or unstimulated cell, STATs are inactive and exists in an unphosphorylated state in the cytoplasm. Receptor

tyrosine phosphorylation occurs upon cytokine stimulation and serves as a docking site for STATs through the SH2 domains leading to reorientation of STAT proteins resulting in the homodimerization or heterodimerization. Once phosphorylated, the dimerized STATs translocate to the nucleus and bind to specific regulatory sequences to activate or repress transcription of target genes. Although the classical JAK-STAT pathway is usually initiated by tyrosine phosphorylation, most vertebrate STATs also contain a second phosphorylation site, which is a serine, and serine phosphorylation also regulates STAT transcriptional activities [202]. STAT1 phosphorylation occurs at Tyr701 resulting in STAT1 dimerization, nuclear translocation and DNA binding. STAT1 can also be phosphorylated at serine (ser727) and serine phosphorylation is required for the maximal induction of STAT1 mediated gene activation. The antibody that I have used in my experiments detects phosphorylation of STATs at Tyr701.

1.3.9.3 NFκB pathway

Nuclear factor kappa B (NFκB) is a family of transcription factors that play important roles in inflammation, immunity, cell proliferation, differentiation and survival. In the inactive state, NFκB is complexed with the inhibitory protein IκBα. IκB kinase complex is activated by inducing stimuli leading to the phosphorylation, ubiquitination and degradation of IκB proteins. The degradation of IκB proteins releases NFκB dimers resulting in their translocation to nucleus where they bind to specific DNA sequences and promote transcription of target genes. NFκB family is composed of five members in mammals such as RelA/p65, RelB, c-Rel, p50 and p52. All the members are characterized by the presence of Rel homology domain (RHD), which is essential for dimerization and binding to cognate DNA elements [203]. NFκB subunits has sites for phosphorylation and other post-translational modifications that are important in the activation and cross-talk with other pathways [204]. NFκB activation occurs in two different

pathways. The canonical activation pathway is mediated through TLRs, Interleukin 1-receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors [205]. Activation through these receptors results in the activation of I κ B kinase complex and leads to the phosphorylation of and consequent degradation of I κ B α . The alternate pathway of NF κ B activation is initiated by different class of receptors including B-cell activation factor (BAFFR), lymphotoxin β receptor and CD40/CD40L interaction [206].

1.3.9.4 Role of signaling pathways in African trypanosomiasis

MAPK and STAT family proteins are important in regulating pro-inflammatory cytokine production in immune cells [207]. Although infection with African trypanosomes leads to profound production of proinflammatory cytokines, the intracellular signaling pathways leading to the production of these cytokines are poorly studied. There are sporadic and inconsistent reports on the role of MAPK and STAT family proteins in Trypanosome-induced proinflammatory cytokine production. Soluble VSG of *T. brucei rhodesiense* has been shown to initiate a cascade of ERK, p38 and JNK MAPK and NF κ B pathways eventually inducing the expression of various pro-inflammatory genes such as TNF, IL-12, IL-6 and iNOS [67]. Consistent with this, several protozoan infections other than African trypanosomes have also been shown to induce the phosphorylation of various MAPKs, leading to enhanced cytokine production. One study shows that ERK and p38 phosphorylation was triggered by *T. cruzi* GPI anchor, leading to the activation of NF κ B and culminating in the activation of pro-inflammatory genes [208]. A recent report shows the *T. cruzi* induced STAT1 phosphorylation both at mRNA and protein level which is associated with increased binding of STAT1 homodimers to gamma activated site (GAS) elements [209]. In addition, MAPK and STAT3 phosphorylation was upregulated in *Toxoplasma gondii* infection [210, 211]. No study has addressed the intracellular

signaling events that lead to pro-inflammatory cytokine release following infection with *T. congolense*. In objective II, I addressed this gap in knowledge and provide strong evidence to show that MAPK and STAT proteins play a critical role in *T. congolense*-induced proinflammatory cytokine production in macrophages.

1.4. Innate immune receptors

The germ line encoded innate receptor families are collectively called as pattern recognition receptors (PRR). They include nucleotide-binding oligomerization domain (NOD)-like receptors (NLR)s, RIG-1-like receptors (RLRs), membrane bound C-type lectin receptors (CLRs) and trans-membrane Toll-like receptors (TLRs).

1.4.1 Toll-like receptors (TLR)

Toll like receptors are a family of trans-membrane receptors and have important role in the recognition of molecular signatures of microbial infection resulting in inflammatory response [176]. Toll like receptors are the first group of pattern recognition receptors identified and they can recognize a wide range of pathogen associated molecular patterns (PAMPs) [212, 213]. By inducing distinct gene expression patterns, stimulation of different TLR leads to the activation of innate immunity, which in turn instructs the development of antigen-specific acquired immunity [214]. Ten human and twelve mouse functional TLRs have been identified till date and each TLR detects distinct PAMPs derived from bacteria, viruses, fungi and parasites [215, 216]. The identification of Toll, which is a receptor expressed by insects (*Drosophila*), was the beginning of the discovery of TLR family. TLRs are type I transmembrane proteins with an ectodomain, transmembrane region and cytosolic domain. The ectodomain mediates the recognition of PAMPs and consists of leucine-rich repeats and the cytosolic Toll-IL-1 receptor (TIR) domain that activate downstream signaling pathways. TLRs recruit specific set of adaptor proteins

MyD88 and TRIF that harbor TIR domain and results in the downstream signaling events that leads to pro-inflammatory cytokine production [217]. This results in the activation of various cells leading to the direct killing of the infected pathogens.

TLRs can be localized on the cell surface (membrane bound receptors) or intracellularly. TLR1, TLR2, TLR4, TLR5 and TLR6 are localized on the cell surface while TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly and majority of intracellular TLRs recognize nucleic acids that are delivered to the intracellular compartment after the uptake of various pathogens [218]. In response to various PAMPs, sequential coordination and dimerization of cytoplasmic TIR domain of TLRs occur, resulting in the recruitment of various adaptor molecules including myeloid differentiation primary response protein 88 (MyD88), TIR domain containing adaptor protein (TIRAP), TIRAP inducing IFN β (TRIF) and TRIF related adaptor molecule (TRAM) [214]. With the exception of TLR3, all TLRs signals through MyD88 and transmits signals resulting in the activation of MAPK and NF κ B, thereby inducing pro-inflammatory cytokine production. TLR3 uses TRIF to activate NF κ B and TLR4 also uses TRIF when the Myd88 independent pathway is activated [219].

Lipopolysaccharide (LPS) from gram-negative bacteria is recognized by TLR4 whereas TLR2 recognizes a wide variety of PAMPs of both gram negative and gram-positive bacteria. TLR2 recognizes lipoproteins and peptidoglycans present in both gram negative and gram positive bacteria and lipoteichoic acid present only in gram positive bacteria [212]. TLR5 recognizes the flagellin protein component of bacterial flagella while bacterial genomic DNA is recognized by TLR9. TLR7 recognizes viral RNA within the lysosomal compartment. It has been shown that microbial pathogens consists of multiple PAMPs which can activate not only TLRS but also other PRRs and it is evident that crosstalk between these receptors is critical for effective innate

and the subsequent adaptive immune responses. The simultaneous activation of multiple TLRs may also occur resulting in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity [220]. Indeed, the activation of a single TLR may not induce a strong immune response as those induced by pathogens where multiple TLRs may be engaged. Therefore, the simultaneous activation of multiple TLRs may be critical for robust immune response in real infections.

1.4.2 Innate immune receptors other than TLRs

Although TLRs play a central role in the initiation of innate immune response against pathogens, other PRRs are involved in PAMP recognition and control of innate immunity. These include membrane bound C-type lectin receptors (CLRs) and cytosolic proteins such as NOD-like receptors (NLRs) and RIG-like receptors (RLRs). CLRs are a large superfamily of membrane proteins that mainly elicit inflammatory responses by recognizing fungal and bacterial PAMPs [216]. RLRs mainly recognize RNA species released into the cytoplasm and play a role in coordinating antiviral program via type I IFN production [221]. NLRs are recently discovered cytosolic pattern recognition family of receptors. They are expressed intracellularly, comprises more than 20 members and respond to various PAMPs to trigger inflammatory responses that send critical danger signal to the immune system [222]. Within the NOD family, Nod1 is ubiquitously expressed whereas Nod2 is expressed only on hematopoietic cells [223, 224]. Signals mediated via Nod2 are coordinated through the adaptor molecule Rip2, which is required for the activation of NF κ B and MAPK that ultimately induces the production of various cytokines and chemokines [223, 225]. Although Nod1 and Nod2 play important roles in recognition of intracellular bacterial infection, due to the functional redundancy with TLRs, deletion of these receptors do not have much impact on the outcome of many infections [226].

Activation of the innate receptors including TLRs and NLRs results in the initiation of inflammatory processes that are regulated via inflammasome formation. Inflammasomes are a group of key signaling platforms consisting of multimeric protein complexes. Inflammasome formation is initiated by the activation of inflammasome sensor molecules that has a NOD like receptor or NLR. Inflammasomes can detect pathogenic microorganisms resulting in the activation of pro-inflammatory cytokines such as IL-1b and IL-18. The multimeric protein complex has an inflammasome sensor molecule, the adaptor protein ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) and caspase-1. The inflammasome formation is triggered following infection or tissue damage and the first step is the formation of a protein complex, which activates caspase-1 and proteolytically activates the pro-inflammatory cytokines IL-1B and IL-18 [227]. Most of the inflammasome sensor molecules contain NOD-like receptor or NLRs such as NLRP1, NLRP3, NLRP6, NLRP7 etc. These sensor molecules detect a broad range of molecular signatures to sense microorganisms and tissue stress. In addition, certain co-receptors are required by some sensor molecules to recognize their ligands or to be stabilized in their activated states.

Although no study has investigated the role of inflammasomes in *T. congolense* infections, their role in *T. cruzi* infection has been reported. It has been shown that inflammasomes are involved in the innate immune response in *T. cruzi* infections and is associated with IL-1 β production in a NLRP-3 and caspase-1 dependent manner. It has also been demonstrated that NLRP3 inflammasome controls *T. cruzi* parasitemia by inducing NO production via caspase-1 dependent and IL-1R dependent pathways [228]. Given that the production of proinflammatory cytokines (including IL-1 and TNF) is a hallmark of African trypanosomiasis, it is conceivable that inflammasomes may play a critical role in this disease.

1.4.3 Innate immune receptors in African trypanosomiasis

Although various TLRs have been shown to play important role in the innate immune responses to several parasitic infections [98, 229, 230] [231, 232], their role (particularly other PRRs distinct from TLRs) has been poorly explored in African trypanosomiasis. The findings that synergistic recognition of *T. cruzi* DNA by TLR9 and glycosylphosphatidyl inositol (GPI) by TLR2 cooperatively leads to induction of proinflammatory cytokine production in macrophages [233] suggests that these TLR may play important role in recognition of African trypanosomes, which is a related organism. Indeed, a recent report showed that, in *T. brucei* infections, TLR2 mediated signaling has been shown to participate in intra-cerebral control of parasite load in the brain [234]. Surprisingly no study has investigated the mechanisms through which the innate immune system detects and responds to *T. congolense* infection. In chapter V of this thesis, I performed studies to fill the gaps in knowledge in this area by assessing the receptors involved *T. congolense* recognition by macrophages and the subsequent intracellular signaling pathways involved in *T. congolense* induced pro-inflammatory cytokine production. I further confirmed the results by infecting different TLR deficient animals with *T. congolense* and monitoring them for parasitemia, survival time and immune response in the liver and spleen.

There are no reports on the role of NLRs in resistance and susceptibility to African trypanosomiasis. However the involvement of NOD receptors in *T. cruzi* infection was reported recently. The major signaling pathways downstream of Nod1 and Nod2 are NF κ B and MAPK pathways and it has been shown previously that in response to *T. cruzi*, IFN- γ primed macrophages trigger NO through NF κ B and MAPK pathways [225, 235]. Recent report suggests that Nod1 dependent responses play a critical role in host resistance against *T. cruzi*

infection [236]. The role of NLRs in resistance to *T. congolense* infection is not studied in the present thesis.

1.5 Trypanocidal agents

1.5.1 Commonly used trypanocidal agents

Chemotherapy is the major means of controlling the impact of African trypanosomiasis on animal health and production in disease prevalent areas. However the development of new anti-trypanocidal drugs has been static over the past three decades due to low research, particularly in the area related to development of new anti-trypanocidal agents [237]. Other complementary measures based mainly on eradication of tsetse fly by using insecticides, traps and sterile insect release are not successful. The most commonly used drugs for treating animal trypanosomiasis include isometamidium, homidium and diminazene aceturate (Berenil). These drugs are used primarily for *T. congolense*, *T. vivax* and *T. brucei* infections. Quinapyramine is the drug mainly indicated for the treatment of *T. evansi* infections. The lack of interest by pharmaceutical industry in the development of new anti-trypanosomal drugs has fueled intensification of research into understanding the mechanisms of action of the existing drugs [237].

1.5.2 Isometamidium and homidium

Both Isometamidium and homidium are phenanthridinium compounds and the anti-trypanocidal activity of these compounds were first demonstrated around 50 years ago [238]. Isometamidium has properties of both homidium and diminazene as it possess an additional moiety of m-amidinophenyl-azo-amine. Both the drugs are used for treatment of *T. congolense*, *T. vivax* and *T. brucei* infections. However, the use of homidium has been reduced due to the widespread resistance [239]. The mechanism of action of these drugs are not fully understood but is thought to be due to its ability to block nucleic acid synthesis by intercalation of DNA base pairs, and

inhibition of RNA polymerase and DNA polymerase [239]. They have also been shown to cleave the kinetoplast DNA minicircles [240].

1.5.3 Suramin

Suramin has been in use since 1920s and is considered the drug of choice for early stages of human African trypanosomiasis. However its mechanism of action is not clearly understood. The drug has been shown to be taken up by pinocytosis as plasma protein-bound complex thereby inhibiting various trypanosomal enzymes [241]. It has been shown that in humans, suramin binds extensively to plasma proteins and persist for a long time (up to 3 months) in circulation [242]. Because it does not cross the blood brain barrier, suramin is ineffective for treatment of advanced stages of the disease when the central nervous system is affected. Although there are ongoing research on the drug's efficacy, mode of action and drug resistance, there are still several unanswered questions. Therefore further study is needed in order to improve the use of these drugs in treatment of trypanosomiasis.

1.5.4 Diminazene aceturate (Berenil)

1.5.4.1 Introduction

For over 60 years, Berenil has been used for the treatment of animal trypanosomiasis. The drug was first introduced into the market as a trypanocide and babesiacide for domestic livestock. Because of its higher therapeutic index and low incidence of resistance compared to other compounds, it became the most commonly used therapeutic agent for trypanosomiasis in livestock. Chemically, it is an aromatic diamidine with two amidinophenyl moieties linked by a triazene bridge [243, 244]. Some of the problems associated with the compound include high cost, risk of drug resistance and disease relapse. In addition, despite its use for more than a century, Berenil's mechanism of action and its effect on the host immune system are poorly

understood. A complete understanding of Berenil's mechanism of action is critical in order to maximize its therapeutic and prophylactic potentials.

Studies have shown that the main biochemical mechanism of Berenil's trypanocidal action is by binding to trypanosomal kinetoplast DNA (kDNA) in a non-intercalative manner through specific interaction with sites rich in adenine-thymine base pairs [245]. This binding results in diskinetoplastic condition with irreversible damage leading to the development of akinetoplast parasites [246, 247]. Berenil can also induce heterochromatin condensation during G2 phase of cell cycle causing the DNA to become completely unfolded [248]. It also interferes with binding of DNA topoisomerase and alters the confirmation of DNA thereby inhibiting DNA replication [249]. Although these biochemical properties contribute to the anti-trypanocidal effects of Berenil, few other reports suggest that the compound has some effect on the host immune system. In *T. congolense*-infected cattle, Berenil treatment at the time of vaccination abolishes immunosuppression and enhances the immune response to *Leptospira biflexa* and reduces immune suppression in *Brucella abortis* challenged animals [250]. Treatment of *T. congolense*-infected highly susceptible BALB/c mice with Berenil leads to control of parasitemia and a dramatic increase in plasma levels of parasite-specific IgG2a and IgG3 antibodies [74]. Another study showed that BALB/c mice cured with Berenil against *T. congolense* are protected against homologous challenge for up to 36 days post challenge [251]. This protection could not be achieved with either Berenil treatment of naïve mice or with anti-serum transfer from infected mice alone. In addition, Berenil has been shown to block histamine-induced responses in tissues and exert some anti-histaminic and anti-inflammatory effects *in vivo* [252]. Taken together, these studies indirectly indicate that in addition to its direct trypanolytic property, Berenil administered during infection has a modulatory effect on the host immune response.

1.5.4.2 Pharmacology

Diminazene has a higher therapeutic index in cattle than any other trypanocide and it is usually effective against infections in cattle resistant to other drugs. The metabolites of Diminazene in animals are not known. Several studies report the recovery of unchanged compound in the liver, feces, urine and milk of treated cattle [253]. Onyeyili et al showed that there was no significant difference in the percentage of Diminazene recovered in the urine of *T. congolense*-infected and healthy dogs over 72 hrs following treatment [254]. In addition, the study concluded that the kidney was the major route of excretion of the drug in dogs [254]. Several pharmacokinetics studies in cattle, rabbits, goats and sheep indicate that following oral administration, absorption occurs immediately leading to rapid attainment of IC50 in the plasma [255]. These studies also show no difference in the time to attain and maintain the peak concentration of the compound in the plasma of uninfected and *T. congolense*-infected cattle [255]. Interesting, there appears to be species-specific differences in the distribution of the Berenil in the plasma with several studies reporting biphasic and triphasic distribution in different animal species [256]. Following intravenous injection in cattle, Berenil penetrates the red blood cells and is distributed extensively to several tissues and organs [256]. In fact, it has been shown that the drug is capable of penetrating several biological and physiological barriers and membranes including the brain, placenta and mammary gland [256]. It has been reported that the degree of uptake of Diminazene by trypanosomes influences its trypanocidal activity and the presence of infection at the time of treatment can significantly deplete the drug concentration in plasma resulting in apparent reduction in the drug's half life [257].

Recently, several studies have shown that Diminazene influences several other physiological conditions. Diminazene has been show to possess an angiotensin converting enzyme II (ACE II)

activating property and thus activates the protective axis of renin angiotensin system (RAS) leading to the cleavage of the octapeptide angiotensin II [258]. ACE II metabolizes angiotensin II (Ang-II) to angiotensin (1-7) and thus counter-regulates the deleterious effects of angiotensin II. These reports suggest a possible role of Berenil in treatment of conditions caused by activation of Ang-II axis. Indeed, a recent study demonstrated that chronic administration of Diminazene prevents and arrests pulmonary hypertension in experimental models of lung injury [259]. Furthermore, increased expression of ACE II mRNA level was observed in Diminazene treated bleomycin and monocrotaline (MCT)-induced pulmonary hypertension rats [259]. Berenil has also shown to have protective effects under various physiological conditions including ischemic stroke and glaucoma [260, 261]. No study has investigated whether Berenil affects Trypanosome-induced proinflammatory cytokine production in infected animals and the molecular mechanisms through it does this. Addressing this huge gap in knowledge forms a major thrust of the studies reported in chapters V and VI in this thesis.

CHAPTER 2. RATIONALE AND HYPOTHESES

2.1 RATIONALE

Despite the fact that African trypanosomiasis is a major health issue to both humans and animals causing vast morbidity and mortality, there is no proper treatment or vaccine available for controlling the disease. The current treatment methods have several limitations and this have stimulated research into control or preventive regimens to serve millions of individuals at the risk of contracting the disease. Hence, there is an urgent need for the development of new therapeutic strategies. Understanding the complexity of immunological mechanisms involved in resistance as well as in the pathogenesis of African trypanosomiasis is essential for the development of effective vaccines and therapeutic treatment strategies.

Because of the low incidence of resistance compared to other compounds, Diminazene aceturate (Berenil) is the most commonly used therapeutic agent for trypanosomiasis. Although it has been in use for almost 60 years, its mechanism of action and effect on the host immune system are poorly understood. Several studies indirectly indicate that Berenil administered during infection has a modulatory effect on host immune response in addition to its trypanolytic activity. It has been shown that Berenil treatment of *T. congolense* infected cattle at the time of vaccination abolishes the parasite mediated immunosuppression and enhances the immune response to *Leptospira biflexa* and *Brucella abortis* [250]. *Uzonna et al* has shown that treatment of *T. congolense* infected Balb/c mice on day 6 and 7 resulted in an increase in the plasma levels of parasite specific IgG2a and IgG3 antibodies, which could be responsible for control of the parasitemia [74]. Another study showed that challenge of Berenil-cured BALB/c mice with homologous variant resulted in an infection with very long prepatent period and control of first peak of parasitemia with prolonged survival time of about 36 days [251]. Collectively these

studies suggest that in addition to clearing the parasites, Berenil has some effect on the host immune response. Therefore in the first part of the thesis, I investigated whether Berenil treatment modulates the host immune response to the parasite in *T. congolense* infected mice. For this I examined several immune parameters of mice infected with *T. congolense* and treated with Berenil.

Many protozoan parasites are recognized at early stages of the infection by the innate immune system through innate immune receptors. It is critical to understand how innate immune system detects and responds to *T. congolense* infection to control or avoid the excessive immune response. The role of Toll-like receptors in initiating the innate immune response has been studied in several parasitic infections. TLR2-mediated signaling has shown to participate in the intra-cerebral control of *T. brucei* infection in brain [234]. In cerebral malaria pathogenesis innate immune responses through TLR2, TLR9 and MyD88 are critically involved [262]. Although it is known that *T. congolense* infection is associated with excessive production of pro-inflammatory cytokines in mice, the signaling pathways leading to the production of these cytokines remain unknown. In the second part of the thesis, I investigated the innate immune receptors; adaptor proteins and signaling pathways associated with *T. congolense*-induced proinflammatory cytokine production.

Berenil is the drug of choice for treating animal trypanosomiasis and although not approved for human treatment, there are reports that it has been used to treat human trypanosomiasis [263, 264]. Preliminary studies from our lab found that Berenil might modulate the host immune response to the parasite in a manner that dampens excessive immune activation and production of pathology-promoting pro-inflammatory cytokines. Therefore in the 3rd part of the thesis, I

investigated whether Berenil down-regulates *T. congolense*-induced MAPK and STAT signaling and cytokine production.

In the first part of thesis I showed that Berenil treatment suppresses proinflammatory cytokine production by splenic and liver macrophages leading to a concomitant reduction in serum cytokine levels in mice infected with *Trypanosoma congolense*. Therefore, in the last part of this thesis, I wanted to further investigate whether the effect of Berenil on pro-inflammatory cytokine production is specific to the parasite, or global. For this, I used LPS and examined whether Berenil down-regulates LPS-induced macrophage pro-inflammatory cytokine production *in vitro* and *in vivo*.

2.2 HYPOTHESES

1. I hypothesized that Berenil has a modulatory effect on the host immune response to *T. congolense* infection.
2. Experimental *T. congolense* infection is associated with excessive pro-inflammatory cytokine production. Therefore, I hypothesized that *T. congolense* induced cytokine production is mediated through MAPK and STAT signaling pathways.
3. Since Berenil treatment down-regulates *T. congolense* induced pro-inflammatory cytokine production, I hypothesized that down-regulation of pro-inflammatory cytokine production by Berenil is by affecting MAPK and STAT pathways.
4. Since Berenil down-regulates *T. congolense* induced pro-inflammatory cytokine production by inhibiting the phosphorylation of MAPK and STAT pathways, I hypothesized that the down-regulation of cytokine production is not parasite-specific and Berenil downregulates LPS induced cytokine production through MAPK and STAT pathways.

2.3 OVERALL OBJECTIVES

There are 4 main objectives in this thesis:

1. To determine how Berenil modulates the host immune response to *T. congolense*
 - a. Investigate whether treatment of infected BALB/c mice with Berenil alters the activation status of lymphocytes and frequency of regulatory T cells.
 - b. Examine whether Berenil treatment affects systemic levels of levels of pro-inflammatory cytokine production in *T. congolense* infected mice.
 - c. Investigate whether Berenil alters the responsiveness of splenic and hepatic CD11b⁺ cells from *T. congolense* infected mice

2. To investigate the molecular mechanisms and signaling pathways associated with *T. congolense*-induced cytokine production
 - a. Examine *T. congolense*-induced cytokine production in macrophages both *in vivo* and *in vitro*
 - b. Elucidate the intracellular signaling pathways and adaptor proteins associated with *T. congolense*-induced cytokine production both *in vivo* and *in vitro*
 - c. Investigate the role of innate immune receptors in *T. congolense*-induced cytokine production
3. To study the effect of Berenil on *T. congolense*-induced cytokine production
 - a. To examine whether Berenil down-regulates *T. congolense*-induced cytokine production in macrophages.
 - b. To investigate the effect of Berenil on *T. congolense*-induced MAPK and STAT pathways in macrophages
 - c. Effect of Berenil on cytokine transcription factors and promoter activity.
4. To investigate the role of Berenil on LPS induced pro-inflammatory cytokine production in macrophages
 - a. To test whether Berenil downregulates LPS induced cytokine production in macrophages.
 - b. To investigate whether Berenil alters TLR expression on macrophages
 - c. To determine the effect of Berenil on phosphorylation on MAPK and STAT signaling pathways upon stimulation with LPS.

CHAPTER 3. MATERIALS AND METHODS

3.1 Mice

Six to eight weeks old female BALB/c and C57BL/6 mice were purchased from the University of Manitoba Central Animal Care Services (CACS) breeding facility or from Charles River Laboratory, Quebec. Female Swiss white CD1 mice were also purchased from University of Manitoba (CACS) and these mice were used to expand the trypanosome stabilates. TLR2^{-/-} and TLR4^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbour ME). MyD88^{-/-} femurs were kindly provided by Dr. Eisenbarth S from Yale School of Medicine. All mice were housed in the specific pathogen free environment at CACS at the University of Manitoba and the experiments were approved by University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

3.2 Parasites

Trypanosoma congolense, Trans Mara strain, variant antigenic type (TC13) was used throughout our study. The origin of this parasite strain has been described previously [79]. For expansion of frozen TC13 stabilates, CD1 mice previously immunosuppressed (48 hr. prior to infection) with cyclophosphamide (0.2 mg/kg) and after 48 hr., infected with freshly thawed *T. congolense* stabilates from liquid nitrogen [79]. Three days after infection, blood was collected from the mice by cardiac puncture. Parasites for infection were purified from the blood by DEAE-cellulose anion-exchange chromatography [265]. Eluted parasites were washed in Tris-saline glucose buffer containing 5% glucose and 10% heat-inactivated fetal bovine serum and counted by hemocytometer, resuspended and used to infect BALB/c mice and C57BL/6 mice. Mice were infected by intraperitoneal injection of 10³ parasites.

3.3 Estimation of parasitemia

A drop of blood was taken from the tail vein of each *T. congolense* infected experimental mouse and was placed on a microscopic slide. Parasitemia was estimated by counting the number of parasites present in at least 10 fields at a x400 magnification of light microscope.

3.4 Preparation of Trypanosoma whole cell extract (WCE)

DEAE cellulose chromatography was used to isolate parasites from infected mice. The isolated parasites were washed and resuspended in PBS at a final concentration of 10^8 /ml. After the isolation trypanosomes were mechanically disrupted by sonication (3-5 cycles of sonication, 5 minutes per cycle) and freeze/thawing several times, aliquoted and stored at -80°C until used. The endotoxin level in the preparation was $<0.005\text{EU}$ as detected by the LAL assay (BioReliance, Rockville, USA)

3.5 LPS

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from DIFCO Laboratories (Detroit, MI). For *in vitro* stimulations of macrophages, LPS was used at a final concentration of $1\ \mu\text{g}/\text{ml}$. For *in vivo* injections, LPS was used a concentration of $5\text{mg}/\text{kg}$.

3.6 Diminazene aceturate (Berenil)

Mice were treated with Berenil (Sigma Adrich, St. Louis MO) intraperitoneally at a concentration of $14\ \text{mg}/\text{kg}$. For *in vitro* experiments, I did a kinetics by stimulating cells with different concentrations of Berenil for different time points and I found that Berenil at a concentration of $10\ \mu\text{g}/\text{ml}$ showed lesser toxicity to the cell and higher cytokine production (appendix 2, 4). Therefore, in all my *in vitro* experiments, I have used Berenil at a concentration of $10\ \mu\text{g}/\text{ml}$.

3.7 Isolation and culture of spleen cells

Mice were sacrificed at different time points as indicated and spleens were collected in 15 ml centrifuge tubes (BD, WR) with complete DMEM. The culture medium used is DMEM supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 5×10^{-5} 2-mercaptoethanol (Invitrogen). After collecting the spleen, they were homogenized using a sterile 15 ml tissue grinder (Fisher) with complete DMEM. The single cell suspension was made by passing the homogenate through a sterile strainer (VWR) and centrifuging at 4 °C for 5 minutes at 1200 rpm. After discarding the supernatant, the pellet was re-suspended in 5 ml of red blood cell lysing buffer (ACK buffer) and incubated for 3-5 minutes at room temperature. After washing the cells two times with 10 ml complete DMEM the pellet was re-suspended in 10 ml complete culture medium. The cells were again diluted in trypan blue and were counted by using a hemocytometer. Four million cells were cultured on to 24 well tissue culture plates (Falcon, VWR) and incubated at 37 °C in a CO2 incubator. After 72 hr, the culture supernatant fluids were collected and stored at -20 °C until assayed for cytokines by ELISA. In some experiments, splenic CD11b⁺ cells were isolated by positive selection using AUTOMACS column and antibodies from Miltenyi (Miltenyi Biotec Inc, Auburn, CA) according to the manufacturer's suggested protocol.

3.8 Isolation of Liver macrophages (kupffer cells)

To isolate kupffer cells, infected or uninfected mice were anesthetized with isoflourane and blood was collected by cardiac puncture. The chest cavity was opened and the liver was perfused by injecting 10 ml ice-cold PBS into the right ventricle. Thereafter, the liver was minced in collagenase solution (1mg/ml), digested at 37°C for 1 hour and passed through a 70-µm-cell strainer (VWR, ON, Canada). Cells were washed with 30 ml Hanks balanced salt solution (HBSS) (Invitrogen, ON, Canada) at 1200 rpm for 5 min. Contaminating red blood

cells were lysed with ACK lysis buffer, washed once with Hank's balanced salt solution (HBSS) and the cells were resuspended in 4 ml 40% percoll (Sigma). Liver lymphocytes were separated by layering the cells on top of 70% percoll (Sigma) and centrifuging at 750 g at 22 °C for 20 min without brakes. The interface containing the mononuclear cells was carefully collected, washed twice with PBS and re-suspended in complete DMEM medium. CD11b⁺ cells were then enriched by positive selection using AUTOMACS (Miltenyi Biotec). Enriched liver CD11b⁺ cells were greater than 96% and were positive for F4/80 expression as assessed by flow cytometry. The cells were washed, counted and cultured for 24 hr in the presence or absence of LPS (1 µg/mg) and culture supernatant fluids were assayed for IL-6, TNF and IL-12 by ELISA.

3.9 Isolation of peritoneal macrophages after treatment with Berenil

Groups of mice were treated with or without Berenil i.p (14 mg/kg). After 24 hours, the mice were either challenged with PBS or LPS (5 mg/kg), sacrificed at different times (3-12 hr) and the peritoneal lavage fluid was collected as previously explained [266]. In brief, mice were sacrificed and sprayed with 70% ethanol. The outer skin of the peritoneum was cut by using scissors and forceps and pulled in back to expose the skin lining the peritoneal cavity. Inject 5 ml of ice cold PBS into the peritoneal cavity using a 27g needle by carefully not puncturing any organs. Gently massage the peritoneum after injection to dislodge any attached cells onto the PBS solution. Insert a 25 g needle to the peritoneum and collect the fluid in a 5 ml syringe and collect the cell suspension in a tube kept on ice after removing the needle from the syringe. The cells in the peritoneal lavage fluid were collected by spinning the suspension for 1250 rpm for 5 minutes. Peritoneal wash fluid is collected from mice inoculated with WCE or live *T. congolense* or mice stimulated with LPS at different time points. Macrophages were isolated

from the peritoneal lavage and either directly lysed with lysis buffer or further stimulated *in vitro* with LPS (1µg/ml) for different time periods. The cells were then routinely lysed and the lysates were used for western blot. In addition, the peritoneal wash fluids were assessed for IL-6, IL-12 and TNF by ELISA.

3.10 Induction of septic shock

To determine the influence of Berenil on LPS-induced septic shock, BALB/c mice were injected with Berenil 24 hr prior to being challenged intraperitoneally with LPS (5 mg/kg). Mice were monitored for movement, body condition and alertness every 3 hr and disease severity was scored in a semi-quantitative fashion as follows: 0, = no abnormal clinical sign; 1, = ruffled fur but lively; 2, = ruffled fur, moving slowly, hunched, and sick; 3, = ruffled fur, squeezed eye, hardly moving, down and very sick; 4, = moribund; and 5, = dead. Clinical score 4 was used as the humane endpoint because the institutional ethical regulation does not permit score 5 in all animal experiments. Mice were sacrificed after 24 hr and peritoneal wash fluid and serum were collected for cytokine analysis.

3.11 Preparation of bone marrow derived macrophages and dendritic cells

Bone marrow-derived macrophages were differentiated from marrow cells as previously described [267]. Briefly, bone marrow (BM) was flushed from the tibia and femur of C57BL/6 mice with complete RPMI using a 10 ml syringe and 30-gauge needle. BM clumps were disrupted by aspirating the cells in and out several times with syringe attached with 21G needle. The BM suspension was spun at 1200 rpm for 5 minutes and the red blood cells were lysed using the red blood cell lysing buffer (ACK buffer). The cells were counted and re-suspended at 4×10^6 and cultured on to the petri-plates. For the differentiation of macrophages, the cells were cultured in complete RPMI with 30% L929 cell culture supernatant (conditioned

medium). After 7 days, the cells were harvested, washed, resuspended in complete medium and used for experiments. The expression of CD11b, F4/80 was assessed by flow cytometry. BMDMs were pre-treated with Berenil for overnight and then stimulated with LPS or WCE and collected the supernatant for cytokine detection and the cells were lysed by NP40 lysis buffer for western blot.

To differentiate bone marrow-derived dendritic cells (BMDCs), marrow cells were seeded in 100 x 15 mm Petri dishes at 2×10^5 /ml in the presence of recombinant murine GM-CSF (20 ng/ml, PeproTech, Rocky Hill, NJ). The culture medium was changed twice on days 3 and 6, and on day 7, the non-adherent cells (dendritic cells) were collected, washed twice with complete medium and used for *in vitro* experiments as described below.

3.12 Cell lines and cell culture

The origin of retrovirus-immortalized bone marrow-derived macrophage cell line (ANA-1) from C57BL/6 mice has been previously described [268]. The immortalized cell lines were grown in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum [Hyclone, Fisher Scientific, Ottawa, Canada], 10 U/ml Penicillin/streptomycin and 50 μ M 2-mercaptoethanol). The cells were cultured in 24-well plates, treated overnight with different concentrations of Berenil (0.01-10 μ g/ml) and then stimulated with LPS (1 μ g/ml) for additional 12-16 hrs. The ANA-1 cells were also used to study WCE induced cytokine production and for that ANA-1 cells were inoculated with WCE (1:10 ratio) for overnight and collected supernatant for cytokine detection. The culture supernatant fluids were collected and stored at -20 $^{\circ}$ C until used for cytokine assays.

3.13 Enzyme Linked Immunosorbent Assay (ELISA)

Sandwich ELISA was performed to determine the cytokine levels in the supernatants collected from cell cultures and serum. ELISA plates were coated with primary antibodies (Biolegend, San Diego, CA) at a concentration of 1-1.2 µg/ml and incubated overnight at 4⁰C. The plates were washed 5 times after ON incubation by using the automated washing machine BIOTEK ELX405 plate washer, Biotek instrument, Winooski, VT) with wash buffer made of 1x PBS and 0.05% Tween 20 at a pH of 7.4. In order to block the non-specific binding, 200 µl of blocking buffer solution made up of 5% new calf serum in PBS at a pH of 7.4 was added to the wells and incubated for 2 hours at 37⁰C after the washing. The plates were incubated for 2 hrs and thereafter washed again with wash buffer. The recombinant cytokine 2ng/ml (standard) was applied on to the plate in dilution buffer for 7 wells and samples were approximately diluted and incubated ON at 4⁰C and the plates were washed 5 times in the ELISA washer. Biotinylated detection antibody at a concentration of 2 µg/ml (Bio-legend) was diluted and was added to all wells and was incubated for 1-2 hrs at 37⁰C. After this step, 30-minute incubation at 37⁰C was carried out with Streptavidin horseradishperoxidase at 1:3000 dilution (BD, Pharmagen, San Jose, CA). The final wash was done for 10 times and thereafter the two-component ABTS substrate was added. After the development of colour, the plates were read (Spectra Max) at 405 nm.

3.14 Bicinchoninic acid (BCA) protein assay

BCA protein assay is a colorimetric detection method for the quantification of total protein. Add gradient volume of standard protein, bovine serum albumin (BSA) (2mg/ml) to each well (6 standards) and samples (5µl/well). Prepare the working solution by mixing reagent A (which is a mix of sodium carbonate, sodium bicarbonate and bicinchoninic acid) and reagent B (4% cupric sulfate) and add 50 µl of the working solution to each standard and sample. After

mixing the wells thoroughly cover the plate and incubate at 37⁰C for 30 minutes. Cool down the plate to room temperature and measure the absorbance at 562 nm in the plate reader (spectra max) and the concentration of protein was calculated from the standard curve.

3.15 MTT assay and assessment of Berenil toxicity to cells

Bone marrow-derived macrophages were grown in complete RPMI medium, treated overnight with different concentrations of Berenil (0.01 - 100 µg/ml) and assessed for cell survival by MTT, Trypan blue exclusion and Propidium iodide assays. The MTT assay was done as described previously [269-271]. Briefly, the cells (in microplates) were treated overnight with different concentrations of Berenil and 20 µl of MTT solution (Sigma, Mississauga, ON Canada) was added to each well and incubated for 4 hr at 37⁰C. Thereafter, the supernatants were discarded and 200 µl of DMSO was added to the wells to dissolve the insoluble formazan crystals. Formazan quantification was performed using an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength. The trypan blue dye exclusion test and propidium iodide assay for cellular viability were performed as explained previously [272] [273].

3.16 Assessment of TLR expression by flow cytometry

The effects of Berenil on TLR expression on macrophages was assessed by flow cytometry. Briefly, differentiated BMDMs were pre-incubated with varying concentrations of Berenil for 12-24hr and stained with PE-conjugated monoclonal antibody (mAb) against murine TLR4 and TLR2 and APC-conjugated mAb against CD14. TLR9 expression was detected by intracellular staining using the FITC-conjugated mAb against murine TLR9. The relative expression of TLR4, TLR2, CD14 and TLR9 was assessed by flow cytometry and quantified by determining the mean fluorescence intensities (MFI).

3.17 Direct ex vivo staining of spleen cells

At sacrifice, single cell suspensions were made from spleens of *T. congolense*-infected mice and cultured 1 ml of 4×10^6 cells on to 24 well plates. After 4 hrs of incubation, The cells were stimulated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) and 500 ng/ml ionomycin (Sigma) and brefeldin A (Sigma) for 4 hours. After harvesting the cells they were transferred into flow cytometry tubes (BD Falcon), and washed in 1 ml FACS buffer by spinning in a centrifuge for 5 minutes at 1200 rpm at 4 °C. The supernatant was discarded and the cells were incubated with FC blocker (50 µl 2.4G2 Hybridoma supernatant) for 15 minutes on ice. For the intracellular cytokine detection, the cells were washed by centrifuging at 1200 rpm for 5 minutes and fixed in 0.5 ml 2% paraformaldehyde (Sigma) and incubated on ice for 15 minutes and washed with 1 ml FACS buffer (PBS containing 0.1% new calf serum and 0.1% sodium azide (NaN₃)). The cells were then incubated on ice for 15 minutes with 0.1% saponin (Sigma) in FACS buffer and this helps to open up the pores on the membranes. A cocktail of flouochrome-labeled antibodies (0.5 µg/tube) (BD Bioscience, Mississauga, Ontario) was made in 0.1% saponin and the final volume in each tube will be 20 µl/tube. After adding the antibody the tubes were incubated for 25 minutes on ice in dark. The cells were washed with saponin containing FACS buffer and then in FACS buffer to allow membrane closure. The cells were resuspended in FACS buffer 300 µl/tube and were acquired using FACS Canto II (BD Bioscience) and analyzed by using Flow Jo, (Treestar, Ashland, OR).

Foxp3 intracellular staining was carried out using the Treg Staining Kit (eBioscience) in accordance with the manufacturer's recommendations. In brief, cells were treated with fixative/permeabilization buffer, washed and intracellular staining was then performed using PE-conjugated anti-Foxp3 antibody, APC-conjugated anti-CD25 and FITC-conjugated CD4.

3.18 Luciferase reporter assay

Dr. Gail Bishop, University of Iowa, generously provided the IL-6 promoter luciferase constructs. ANA-1 cells in complete RPMI medium were seeded into 24 well plates and at 70-80% confluency, transient transfection of the cells was performed by using Turbofect (Fermentas Canada Inc, Ottawa, ON, Canada) according to the manufacturer's suggested protocol. Into each well, cells were co-transfected with 0.8 µg IL-6 promoter luciferase DNA vector and 0.2 µg Renilla luciferase vector pRL-TK for 24 hrs. The cells were then washed and treated with Berenil (10 µg/ml) and LPS (1 µg/ml) for 12 hr. Following additional washings, the cells were harvested in passive lysis buffer and the cell lysates were collected. Luciferase activity was assessed by a luminometer using the Dual Luciferase Assay System Kit (Promega, Madison, WI) according to manufacturer's suggested protocol. Luciferase activity was recorded after mixing 20 µl of cell lysate with 100 µl of Luciferase Assay Reagent II and firefly. Followed by this 100 µl of Stop-and-Glo reagent was added and measured the Renilla luciferase activity. All values were normalized to Renilla luciferase activity and expressed relative to transfected unstimulated control cells.

3.19 Western blotting to assess MAPK and STAT3 phosphorylation

Assessment of MAP kinases, STATs and NFκB p65 subunit phosphorylation and the induction of SOCS1 and SOCS3 were determined by western blot as previously described [274]. Briefly, fully differentiated BMDMs were grown in petriplates and once confluent, the cells were serum starved for about 24-48 hrs. The cells were then treated with Berenil (10 µg/ml) overnight and stimulated with LPS (1 µg/ml) or inoculated with WCE for different time points as indicated. Thereafter, the cells were washed twice with ice cold PBS, lysed with NP40 lysis buffer supplemented with a cocktail of protease inhibitors (2mM sodium orthovanadate, 1mM

phenyl-methylsulfonylfluoride) (Sigma-Aldrich) and the total proteins were extracted. Harvested lysates were centrifuged for 10 min at 4⁰C to remove cellular debris. The concentration of protein in the lysate was determined by BCA protein assay. Ten (10) µg of protein lysate was resolved in 10% SDS-PAGE, transferred onto a nitrocellulose membrane, blocked with 5% BSA and probed overnight at 4⁰C with primary antibodies (rabbit anti-mouse mAb) specific for SOCS1 and SOCS3, phosphorylated ERK, p38, JNK, STAT1, STAT3, STAT5 and NFκB p65 subunit. The blots were washed rigorously with TBST, incubated with anti-rabbit HRP conjugated secondary antibody and the bands were revealed by ECL (Amersham, GE Healthcare Biosciences, Pittsburgh, PA) reagents. Thereafter, the blots were routinely stripped and total ERK, p38, JNK, STAT1, STAT3, STAT5 and NFκB p65 subunit were detected and used as loading controls. In addition, the membranes were also stripped and probed for β-actin as additional loading control. Densitometric analysis was performed and the integrated density values were presented as ratio of phosphorylated over the total protein compared with media control.

3.20 Quantification of phosphorylated and total NFκB p65

NFκB p65 phosphorylation was detected by using Fast Activated Cell-based ELISA NFκB p65 profiler kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's suggested protocols. Briefly, the cells were cultured in 96 well plates and stimulated with Berenil (10 µg/ml) overnight and then with LPS (1 µg/ml). At the indicated times, the cells were fixed rapidly, washed and incubated with phospho and total NFκB p65 primary antibody for overnight at 4 °C. The plates were then washed, incubated with HRP-conjugated secondary antibody, developed and the relative number of cells in each well was determined by using crystal violet. The phospho and total NFκB p65 signals were normalized for corresponding cell

numbers and the ratio of phosphorylated NFκB p65 to total NFκB p65 over the controls were determined.

3.21 MAPK and STAT Inhibitors

The p38 MAPK inhibitor, SB-203580 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine, the JNK inhibitor, SP-600125 (anthra[1-9-cd]pyrazol-6(2H)-one), and the p42/p44 ERK inhibitor, U-0121(1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene), were purchased from Calbiochem (Mississauga, Ontario, Canada). Fludarabine (specific inhibitor of STAT-1) was obtained from Sigma-Aldrich (Mississauga, Ontario, Canada). STAT3 inhibitor 2-Hydroxy-4(((4-methylphenyl)sulfonyloxy)acetyl)amino)-benzoic acid (S31-201) was obtained from Santa Cruz Biotechnology (Dallas, USA). The cells were pretreated with the corresponding inhibitors at various concentrations 10 μM-100 μM for 1hr and then inoculated with Trypanosoma WCE and collected the lysate after ON incubation for the detection of cytokines.

3.22 Statistical analysis

Cytokine and densitometric data are presented as mean +/- standard error of mean (SEM). A two-tailed Student's t-test was used to compare differences in cytokine production. Two way analysis of variance (ANOVA) was used to compare differences in phosphorylation between different groups after densitometry. Significance was considered if $p < 0.05$. All analyses were carried out using GraphPad Prism software.

CHAPTER 4. RESULTS

4.0 Diminazene aceturate (Berenil) modulates the host cellular and inflammatory responses to *Trypanosoma congolense* infection.

4.1 Introduction

African trypanosomiasis are diseases of humans and livestock caused by several species of flagellated single-celled protozoan parasites belonging to the genus *Trypanosoma*. Trypanosomes are transmitted from infected to uninfected animals by different species of tsetse fly during regular blood meals. They remain in the bloodstream as extracellular parasites and therefore are constantly exposed to the host's immune system. As a result, African trypanosomes have developed sophisticated immune evasion mechanisms including antigenic variation [81, 275], excessive activation of the complement system leading to hypocomplementemia [81, 276], polyclonal B cell activation [277] and immunosuppression [10]

Trypanosomiasis in animals is caused by *Trypanosoma congolense*, *Trypanosoma brucei brucei* and *Trypanosoma vivax*, and is much the same disease in livestock as it is in humans. The cattle industry in many African countries is hit particularly hard; it is estimated that the disease costs \$1.3 billion to livestock producers and consumers every year [278]. Of the three species of animal trypanosomiasis, *T. congolense* is the most important disease for livestock [279]. While other species, particularly *T. brucei*, has the capacity to invade the capillary interstitial walls, *T. congolense* are purely intravascular and hence unable to leave the circulation [279]. Thus, they are continuously exposed and interact with the host circulatory defense factors leading to extreme immunopathology.

BALB/c mice are highly susceptible to experimental *T. congolense* infection and succumb to the infection within 8-10 days [77]. Death of infected BALB/c mice is usually related to immune hyper-activation of cells, particularly macrophages and T cells, leading to massive production of pro-inflammatory cytokines (including IFN- γ , IL-1, IL-6, IL-12 and TNF- α and systemic inflammatory response syndrome (SIRS) [280]. In contrast, C57BL/6 mice are considered relatively resistant to *T. congolense* infection because they can control several waves of parasitemia and survive for over 100 days [280]. These mice produce low levels of pathology-inducing pro-inflammatory cytokines and their immune cells are relatively quiescent or hypo-activated [280]. Complement and antibody-mediated phagocytosis by splenic and liver (Kupffer cells) macrophages is one of the primary mechanism by which trypanosomes are cleared from an infected host [175]. However, these cells also contribute to the excessive production of pro-inflammatory cytokines following their interaction with the parasites [175].

Chemotherapeutic agents used for treatment of animal trypanosomiasis include dimiazene acetate (Berenil), isometamidium, homidium and suramin. Berenil has been in use as an anti-trypanosome drug for livestock since 1955. The main biochemical mechanism of Berenil's trypanocidal actions is thought to be by binding to kinetoplast DNA [247] thereby inducing complete and irreversible loss of kDNA in certain strains of trypanosomes [246, 281]. Due to its molecular structure, Berenil has particular affinity for A-T base pairs in circular DNA and kinetoplast DNA [245, 247, 282]. Berenil is not licensed for use in humans because of serious side-effects observed in animals, which include tremors, itching, sweating, convulsions, dyspnea, recumbency and vomiting in camels [246] and decreased blood pressure [283] and diarrhea in dogs.

Despite its use for over 60 years, few studies have investigated the ability of Berenil to modulate the host immune responses. Plasma from Berenil-treated cattle showed significant *in vitro* anti-trypanosome activity for up to 3 weeks after a single intramuscular injection, and mice treated with Berenil before infection are protected against homologous challenge up to 42 days post treatment [284]. It has been shown that treatment of *T. congolense*-infected BALB/c mice with Berenil alters the nature of their B cell (antibody) responses, increasing protective IgG2a and IgG3 responses against VSG and whole parasite [74]. In addition, Berenil treatment also abolishes *T. congolense*-induced immunosuppression *in vitro* and *in vivo*, allowing the animals to mount successful immune responses against secondary challenge with a different pathogen [250]. Tabel and Otesile [251] showed that BALB/c mice cured of *T. congolense* infection with Berenil and challenged with a homologous strain of the parasite could control infection for up to 36 days post challenge. This response could not be achieved with either serum transfer from infected mice alone or with Berenil treatment of naïve (uninfected) mice. Furthermore, the isolated parasites from BALB/c mice after challenge were found to be a different variant from the injected strain and mice could not control challenge with a heterologous strain. Taken together, these studies indirectly suggest that Berenil administered during infection modulates the host immune response.

In this aim of the thesis, I examined several immune parameters of mice infected with *T. congolense* and treated with Berenil to investigate whether the drug modulates the host immune response to the parasite. I show that Berenil treatment reduced serum levels of pro-inflammatory cytokines, and alters the activation status of lymphocytes in the spleens and livers of infected mice. These effects could augment the trypanolytic activities of the compound leading to more effective parasite and disease control.

4.2 Results

4.2.1 Treatment with Berenil prevents early death of infected BALB/c mice and alters the activation status of lymphocytes and frequency of regulatory T cells in spleens of infected mice.

BALB/c mice infected with 10^3 *Trypanosoma congolense* are unable to control their first wave of parasitemia and die acutely with mean survival time of 8 ± 1 day [74]. Treatment of infected mice with Berenil (14 mg/kg i.p.) on day 5 post-infection led to clearance of parasitemia by day 7 post-infection and an indefinite survival.

The susceptibility of BALB/c mice to *T. congolense* has been associated with immune cell hyper-activation particularly T cells and macrophages [285]. Furthermore, recent reports suggest that regulatory T cells play important roles in the pathogenesis of *T. congolense* infection in mice [10, 285, 286]. Therefore, I investigated the effects of Berenil treatment on CD25 expression (an activation marker) on lymphocytes and FoxP3 expression (regulatory T cell marker) on CD4⁺ T cells from spleens of treated and untreated mice. There was a marked (50%) reduction in CD25 expression on total lymphocytes from Berenil-treated BALB/c mice (Figure. 4.2.1A). The reduction in the percentage of CD4⁺CD25⁺ cells though significant was not as pronounced as that seen for total lymphocytes (Figure 4.2.1B), suggesting that most of this change was in another lymphocyte population. Furthermore, Berenil-treated BALB/c mice had significantly lower numbers of CD4⁺CD25⁺FoxP3⁺ expressing cells than untreated mice (Figure 4.2.1 C). Similar results were also obtained in infected and treated relatively resistant C57BL/6 mice (Figure 4.2.1D-F). Interestingly, Berenil treatment did not alter the frequency of CD4⁺CD25⁺FoxP3⁺ cells in the spleens of uninfected mice suggesting that the effect

observed in infected mice may be related to changes in the dynamics of Tregs due to *T. congolense* infection (appendix 1) [10, 285, 286]

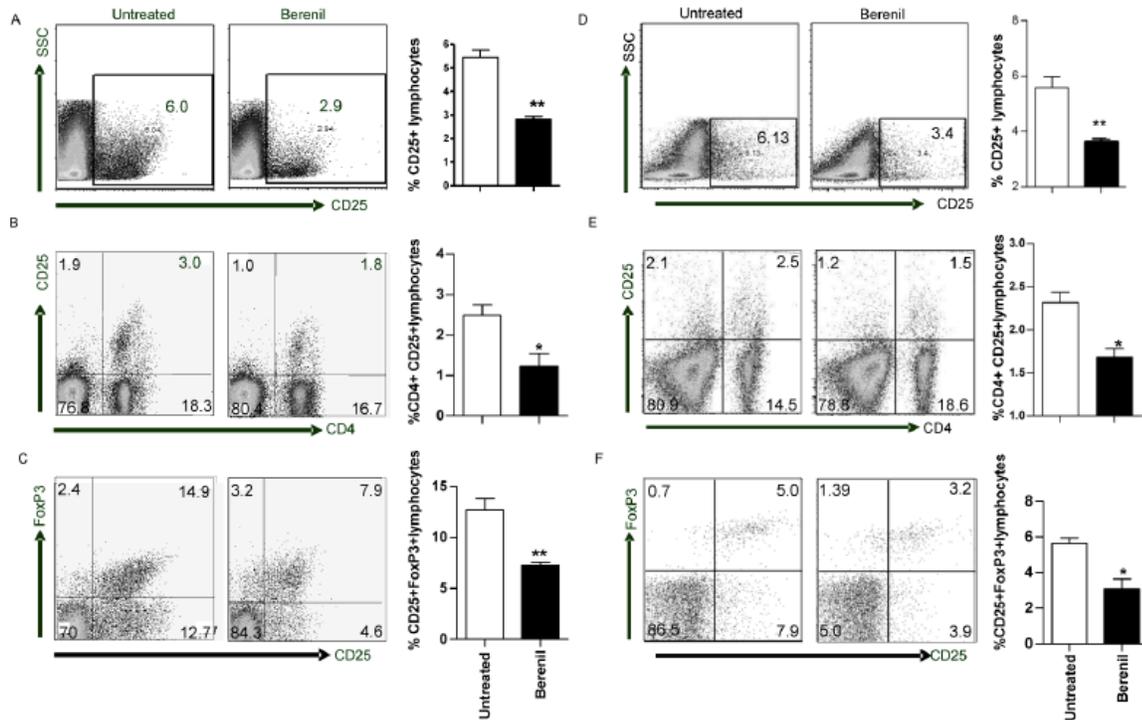


Figure 4.2.1. Berenil treatment decreases the percentage of CD25⁺ and FoxP3⁺ cells in the spleens of infected mice. Splenocytes from *Trypanosoma congolense*-infected BALB/c and C57BL/6 mice either treated or untreated with Berenil were routinely stained directly *ex vivo* with fluorochrome-conjugated mAb against CD4, CD25 and Foxp3 and analyzed by flow cytometry. Shown are representative dot plots showing expression of CD25 on total (A and D) and CD4⁺ (B and E) lymphocytes. Representative dot plot of CD25⁺ and Foxp3⁺ cells gated on CD4⁺ lymphocytes (C and F). The bar graphs represent the cumulative percentages of CD25⁺ and Foxp3⁺ cells from 3-5 mice per group. The results presented are representative of 4 different experiments with similar results. Bars show mean +/-SEM; *, p < 0.05; **, p < 0.01.

4.2.2. Berenil treatment reduces systemic levels of pro-inflammatory cytokines in *T. congolense*-infected mice.

Acute death of *T. congolense*-infected BALB/c and IL-10R deficient C57BL/6 mice is usually attributed to excessive production of inflammatory cytokines by immune cells leading to a cytokine storm and concomitant systemic inflammatory response syndrome [10]. Therefore, I investigated whether Berenil treatment was also associated with reduction in the production of inflammatory cytokines. The levels of several pro-inflammatory cytokines (IL-6, TNF, IL-12, and IFN- γ) were significantly reduced (by several fold) in Berenil-treated BALB/c mice (Figure 4.2.2A-D). This reduction was most dramatic for IFN- γ , which was below detectable levels in the treated group (Figure 4.2.2D). Similar results were also obtained in infected and treated C57BL/6 mice (Figure 4.2.2E-H), suggesting that the effect of Berenil is not mouse strain specific. Paradoxically, Berenil treatment also caused a significant reduction in serum levels of IL-10 in infected BALB/c mice (348 ± 46 pg/ml vs. 56 ± 23 pg/ml, $p < 0.03$ for untreated vs. treated groups, respectively). Interestingly, Berenil treatment of naïve (uninfected) BALB/c mice also caused significant reduction in serum levels of IL-6 (Figure 4.2.2 I) and TNF- α , (Figure 4.2.2 J) although IL-12 and IFN- γ were below detectable levels. These results suggest that the reduction in serum levels of pro-inflammatory cytokines in *T. congolense*-infected mice was not solely due to destruction of parasites and subsequent reduction in parasitemia by Berenil.

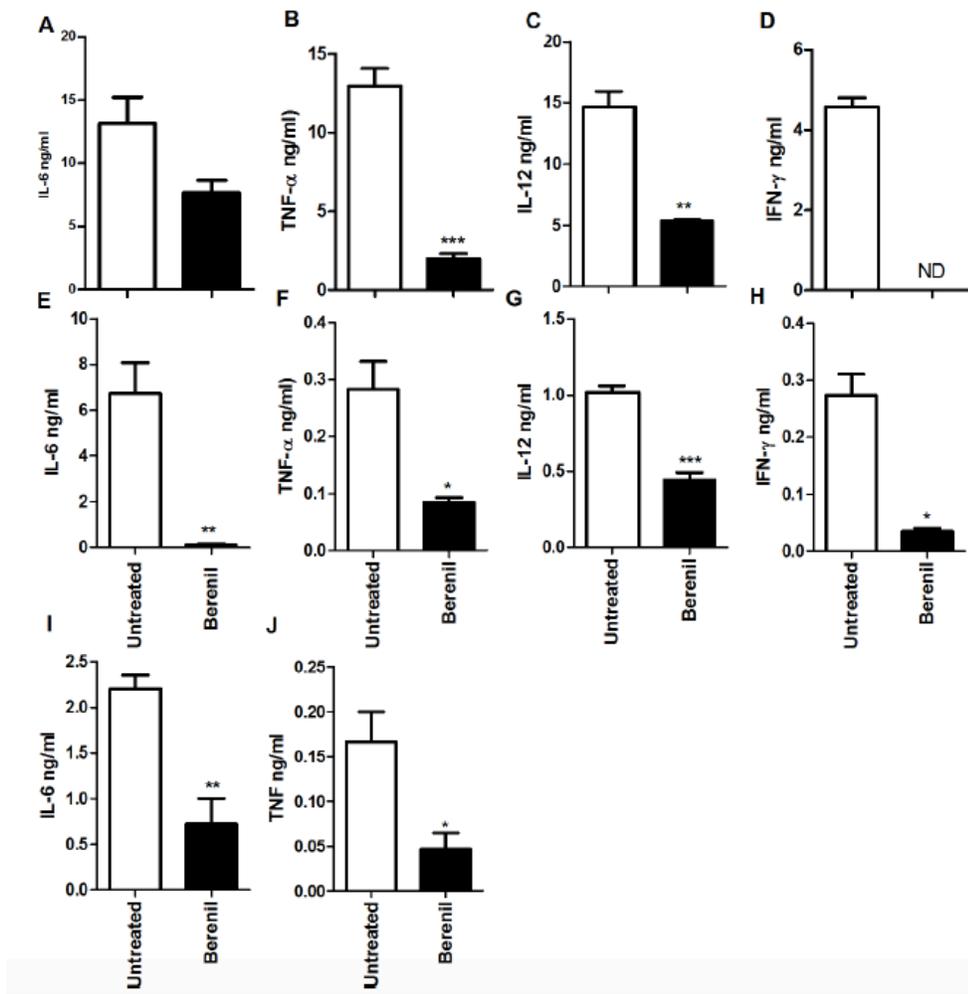


Figure 4.2.2. Treatment with Berenil reduces serum pro-inflammatory cytokine levels in *T. congolense*-infected mice. BALB/c (A-D) and C57BL/6 (E-H) mice were infected with *T. congolense* and treated with Berenil on day 5 post-infection. Eight days post-infection (3 days after Berenil treatment), mice were sacrificed and blood samples were taken via cardiac puncture to obtain serum. The levels of IL-6 (A and E), TNF (B and F), IL-12 (C and G) and IFN- γ (D and H) in the serum were determined by sandwich ELISA. Uninfected BALB/c mice were also treated with Berenil, sacrificed 3 days later and serum levels of IL-6 (I) and TNF (J) were determined by ELISA. The results presented are representative of 3 (A-H) and 2 (I and J) independent experiments (n = 4 mice per group) with similar results. Bars show mean \pm SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001. N.D. = not detected (i.e. below 15 pg/ml).

4.2.3 Berenil treatment alters the responsiveness of splenic and hepatic CD11b⁺ cells to LPS stimulation

Because of their role in phagocytosis and production of inflammatory cytokines, CD11b⁺ cells, particularly macrophages are vital for both clearance of *T. congolense* and in mediating immunopathology in infected mice [175]. Therefore, I examined the influence of Berenil on *ex vivo* purified CD11b⁺ cells from spleens of *T. congolense* infected or uninfected mice following *in vitro* stimulation with or without LPS (5µg/ml). Interestingly, the absolute numbers of CD11b⁺ cells from spleens of Berenil-treated mice were significantly higher than those from untreated mice (106.3 ± 14.3 vs. 66.7 ± 5.5, p = 0.011 for Berenil-treated and untreated animals, respectively). This suggests that alteration in numbers of CD11b⁺ cells is not responsible for the reduction in serum levels of pro-inflammatory cytokines observed in Berenil-treated mice (Figures 4.2.3A-H). Berenil treatment significantly (p < 0.05) suppressed spontaneous as well as LPS-induced production of IL-6, TNF and IL-12p40 by splenic CD11b⁺ cells from infected BALB/c and C57BL/6 mice (Figure 4.2.3 A-F). Similar results were also obtained from CD11b⁺ cells from uninfected mice (Figure 4.2.3G and H), although IL-12 production by cells from uninfected mice was below detectable levels.

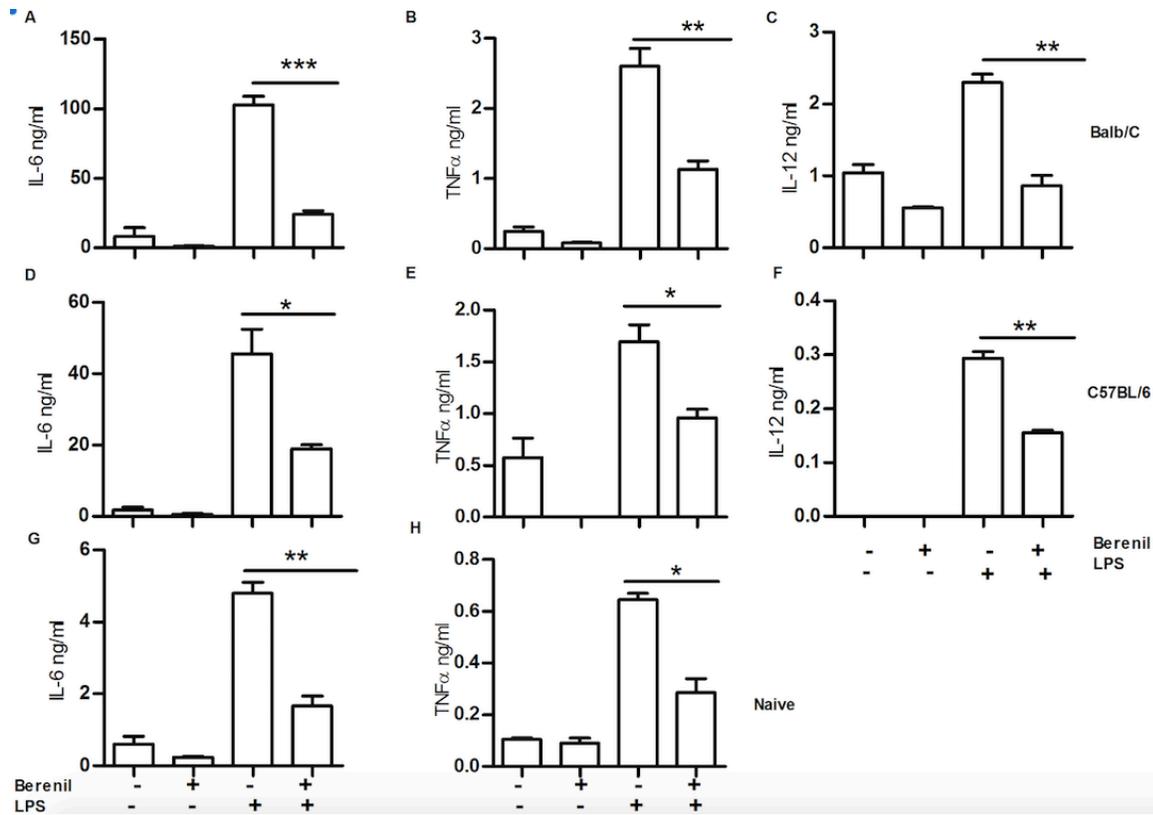


Figure 4.2.3. Berenil treatment suppresses IL-6, IL-12 and TNF production by CD11b⁺ spleen cells from *T. congolense* infected mice. Highly enriched (by positive selection) CD11b⁺ splenocytes from Berenil treated or non-treated infected (A-F) and uninfected (G and H) mice were cultured for 18 hours with or without LPS (5µg/ml) and the culture supernatant fluids were assayed for IL-6 (A, D and G), TNF (B, E and H) and IL-12p40 (C and F) by ELISA. Top (A, B and C) and middle (D, E and F) panels are data obtained with splenocytes from infected BALB/c and C57BL6 mice, respectively. The bottom panel (G and H) shows data from uninfected (naïve) C57BL/6 mice. The results presented are representative of 3 independent experiments (n = 4 mice per group) with similar results. Bars show mean +/-SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001. N.D. = not detected (i.e. below 15 pg/ml)

4.2.4 Berenil treatment reduces pro-inflammatory cytokine secretion by Kupffer cells from *T. congolense*-infected mice

Because clearance of trypanosomes in infected mice is mediated primarily by liver macrophages [175], I isolated kupffer cells from livers of infected mice at Day 8 post-infection and assessed their production of cytokines directly *ex vivo*. I chose to look at Day 8 post-infection because previous report has shown this time to be the peak of kupffer cell activity and maximum parasite uptake after *T. congolense* infection [175]. As shown in Figure 4.2.4A and B, Berenil treatment significantly reduced the spontaneous (directly *ex vivo*) IL-6 and TNF production by kupffer cells from infected mice. Similar effects were also seen for IL-12p40 production although this was not statistically significant (Figure. 4.2.4 C). Taken together, these findings suggest that Berenil can specifically alter macrophage responses *in vivo* by reducing their ability to respond to certain pathogen-derived stimuli.

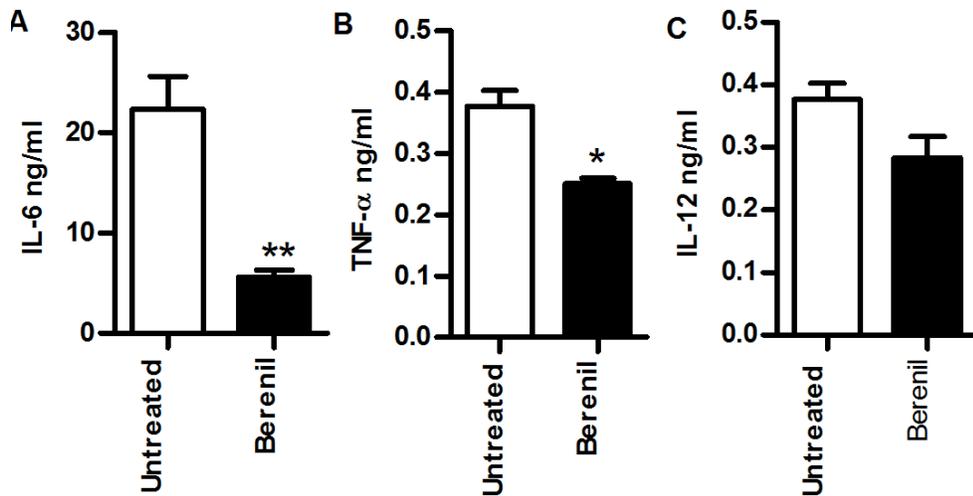


Figure 4.2.4. Berenil treatment reduces spontaneous pro-inflammatory cytokine secretion by kupffer cells from *T. congolense*-infected mice. BALB/c mice were infected with *T. congolense* and treated with Berenil on day 5 post-infection. Eight days post-infection (3 days after Berenil treatment), mice were sacrificed and hepatic mononuclear cells were isolated by Percoll gradient centrifugation. Kupffer cells were further enriched by positive selection using CD11b-coated beads, cultured for 24 hr and the concentration of IL-6 (A), TNF (B) and IL-12 (C) in culture supernatant fluids were measured by ELISA. The results presented are representative of 2 independent experiments (n = 4 mice per group) with similar results. Bars show mean +/-SEM; *, p < 0.05; **, p < 0.01

4.2.5 Berenil ameliorates LPS-induced systemic inflammatory response syndrome

The preceding results suggest that Berenil might also alter the overall host inflammatory response to microbial stimuli. Therefore, I assessed the effects of Berenil on LPS-induced model of septic shock. As shown in Figure 5A-D, pretreatment of mice with Berenil significantly ameliorated LPS-induced toxicity as evidenced by reduction in the overall disease score (Figure 4.2.5A) and significant reduction in serum levels of IL-6 (Figure 4.2.5B) and TNF (Figure 4.2.5C). Berenil treatment also lowers serum levels of MCP-1 although this was not statistically significant (Figure 4.2.5D). Collectively, these findings show that Berenil dampens systemic inflammatory response by altering responsiveness of immune cells to microbial stimuli.

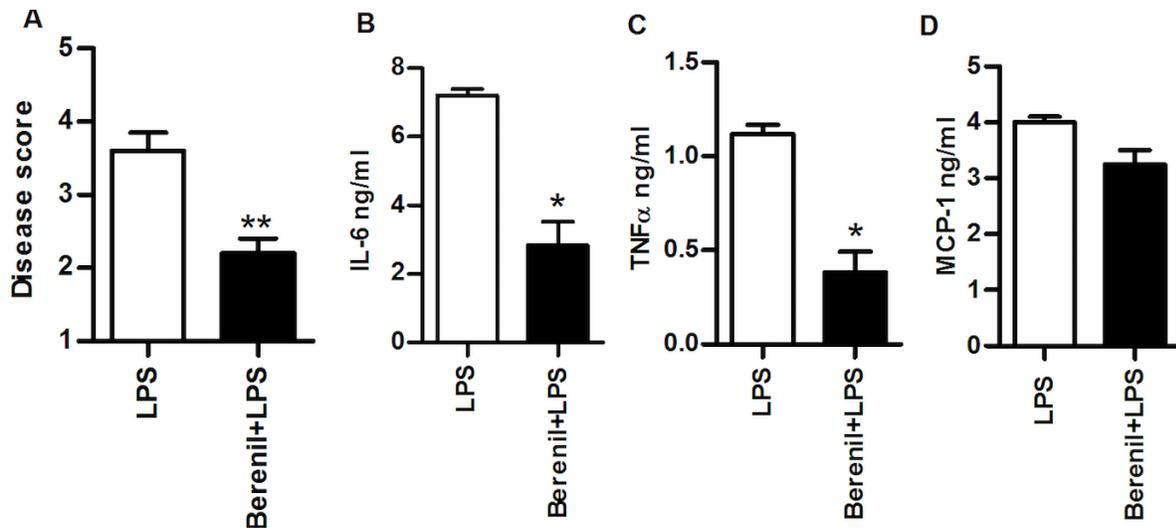


Figure 4.2.5. Berenil ameliorates LPS-induced toxicity and production of pro-inflammatory cytokines *in vivo*. Naïve BALB/c mice were injected with Berenil and after 24 hr challenged with LPS (10 mg/kg) intraperitoneally. After 24 hr, mice were assessed for clinical (disease) score (A), sacrificed and serum levels of IL-6 (B), TNF (C), and MCP-1 (D) were determined by ELISA. The results presented are representative of 2 independent experiments (n = 4 mice per group) with similar results. Bars show mean +/-SEM; *, p < 0.05.

CHAPTER 5

5.0 *Trypanosoma congolense* induced pro-inflammatory cytokine production is by the activation of TLR signaling system through MAPK and STAT proteins.

5.1 Introduction

African trypanosomiasis, an infectious disease of humans and animals, is caused by several species of protozoan parasites belonging to the genus *Trypanosoma*. Because of minimal research in treatment and control measures, African trypanosomiasis is considered as one of the neglected diseases along with other parasitic diseases such as Schistosomiasis, Leishmaniasis, Chagas disease etc. [287]. African animal trypanosomiasis is primarily caused by *T. congolense*, *T. vivax* and *T. brucei brucei* and presents as a mild disease in wild animals but very fatal in domestic animals if untreated. Animal trypanosomiasis has a severe economic impact and affects the agricultural development as it adversely affects livestock production and farming. *Trypanosoma congolense* is the most important African trypanosome and causes debilitating acute and chronic disease in cattle and other domestic animals. Because the parasites are purely extracellular but intravascular, they are unable to leave the circulation and therefore are constantly exposed to the host's immune system. As a result, they have developed sophisticated evasion mechanisms including antigenic variation of the variant surface glycoprotein (VSG) [288, 289], polyclonal B-lymphocyte activation [290] and induction of immunosuppression [52, 96, 250].

Toll like receptors (TLRs) are a family of type 1 trans-membrane receptors and activation of these receptors in turn activates various signal transduction pathways that lead to the induction of various genes including inflammatory cytokines. TLRs have an important role in recognition of molecular signatures of microbial infection, propagate various signaling

pathways and direct the adaptive immune response [176]. The extracellular leucine-rich repeats of the TLRs are responsible for the recognition of pathogens and cytoplasmic region known as Toll/interleukin-1 receptor (TIR) domains are responsible for initiating intracellular signaling pathways [214]. TLR-mediated intracellular signaling events are initiated by heterophilic interactions of TIR-domain with TIR-domain containing cytosolic adaptor proteins such as myeloid differentiation primary response protein 88 (MyD88), TIR-domain containing adapter protein (TIRAP), TIR domain-containing adapter inducing IFN β (TRIF) and TRIF-related adaptor molecule (TRAM) [177, 214]. The binding of TLRs with corresponding ligands lead to the recruitment of a number of adaptor proteins and MyD88 is the major adaptor protein that is a part of almost all TLR signaling pathways. Individual TLRs interact with different combinations of adaptor proteins resulting in the activation of various transcription factors like nuclear factor (NF)- κ B, Activating protein-1 and induces a specific immune response. Members of both mitogen-activated protein kinases (MAPKs) and signal transducer and activator of transcription (STAT) family members can interact to activate multiple intracellular signaling pathways that lead to increased cytokine production.

Mice are the most common animal models for experimental African trypanosomiasis and have provided great insight into the immunopathogenesis of the disease. Balb/c mice are highly susceptible to experimental *T. congolense* infection because they are unable to control the first wave of parasitemia and die within 8-10 days. In contrast, C57BL/6 mice are relatively resistant to infection and can control several waves of parasitemia and survive over 100 days. It has been shown that death of infected animals is due in part to hyper-activation of immune cells (particularly macrophages and T cells) resulting in excessive production of pro-inflammatory cytokines (including IFN- γ , IL-6, IL-12 and TNF), which leads to systemic

inflammatory response like syndrome [74]. However, the innate receptors, adaptor proteins and signaling pathways involved in the *T. congolense* induced cytokine production in macrophages are not known.

In the preceding chapter, I showed that Berenil treatment reduced serum levels of pro-inflammatory cytokines and alters the activation status of lymphocytes in *T. congolense*-infected mice. In this chapter, I investigated the innate receptors involved in *Trypanosoma congolense*-recognition in macrophages, the role of MyD88, and the intracellular signaling molecules like MAPKs and STATs involved in *T. congolense*-induced cytokine production. I show that *T. congolense*-induced cytokine production is dependent on MyD88-mediated activation of MAPKs (ERK, p38, JNK) and STATs (STAT1 and STAT3) signaling and that TLR2 is the critical receptor involved in parasite recognition and induction of resistance in infected mice.

5.2 Results

5.2.1 *Trypanosoma congolense* (TC) induces pro-inflammatory cytokine production in macrophage cell lines and bone marrow derived macrophages (BMDM).

Infection of mice with African trypanosomes (including *T. congolense*) is associated with high levels of serum pro-inflammatory cytokines including IL-12, IL-6, TNF and nitric oxide (NO [291, 292]. Indeed, death of *T. congolense*-infected mice is due to cytokine storm leading to systemic inflammatory response like syndrome (SIRS). However, the key innate immune cells and molecular mechanisms underlying recognition of *T. congolense* are not yet studied. Because macrophages play a central role in resistance to experimental African trypanosomiasis [68, 171], I investigated their role in proinflammatory cytokine production and the molecular pathways involved in this process. First, I stimulated the immortalized macrophage cell line (ANA-1) with *Trypanosoma congolense* whole cell extract (WCE) for 12-16 hrs and assessed cytokine production by ELISA. WCE stimulation induced IL-6, IL-12, TNF, MCP-1 and IL-1 β production in ANA-1 cells (Figure 5.2.1 A-E). I also confirmed the induction of these cytokines in primary macrophages (BMDMs, Figure 5.2.1 F-J), suggesting that WCE-induced proinflammatory cytokine production in macrophages was real and not due to the immortalization process in the ANA-1 cells.

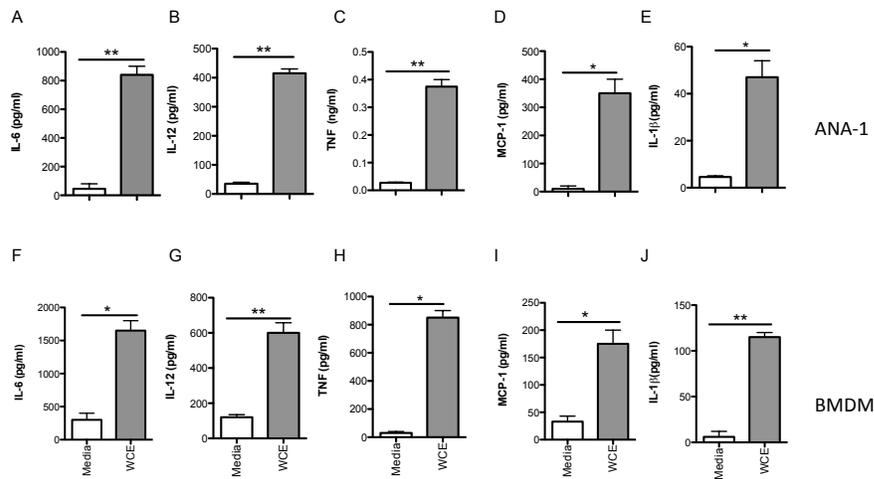


Figure 5.2.1 *Trypanosoma congolense* induces cytokine production in macrophage cell lines and bone marrow-derived macrophages (BMDM).

ANA-1 cells and BMDMs were stimulated with *Trypanosoma congolense* (whole cell extract, WCE, 1:10 ratio) and after 24 hrs., the culture supernatant fluids were collected and assayed for cytokines by sandwich ELISA. Shown is IL-6 (A and F), IL-12 (B and G), TNF (C and H), IL-1b (D and I) and MCP-1 (E and J) levels in the culture supernatant fluids of ANA cells (A-E) and BMDM (F-J). The data presented are representative of three independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$.

5.2.2 *T. congolense* induces phosphorylation of MAPKs and STATs in macrophages.

MAPK and STAT family of signaling molecules are important in regulating pro-inflammatory cytokine production in immune cells including macrophages [207]. Although not yet demonstrated in *T. congolense* infection, several parasitic infections have been shown to activate the MAPK and STAT signaling pathways in infected cells [210, 293]. Therefore I wished to determine whether the induction of proinflammatory cytokine production in macrophages by WCE involves activation of the MAPK and STAT pathways. I stimulated BMDMs with WCE and at different times, assessed the phosphorylation of several MAPK and STAT proteins by western blot. As shown in Figure 5.2.2 A-E, WCE induced the phosphorylation of ERK, p38, JNK, STAT1 and STAT3 proteins in BMDMs at various time points after stimulation. Collectively, these results suggest that *T. congolense*-induced cytokine production could be mediated through MAPK and STAT signaling pathways.

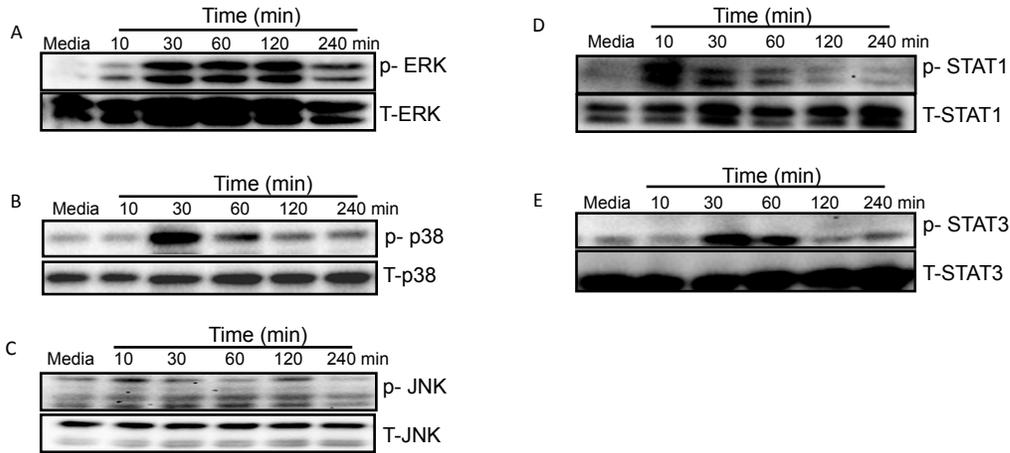


Figure 5.2.2 *T. congolense* induces phosphorylation of MAPKs and STATs in macrophages. BMDMs were stimulated with endotoxin-free WCE and at indicated times, the cells were lysed and lysates were assessed by western blot for phosphorylation of ERK (A), p38 (B), JNK (C), STAT1 (D) and STAT3 (E) proteins using the appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38, JNK, STAT1 and STAT3 and used as loading controls. The data presented is a represent three independent experiments with similar findings.

5.2.3 Inhibitors of MAPKs and STATs abolish *T. congolense*-induced cytokine production in macrophages

To determine the involvement of MAPKs and STATs in *T. congolense*-induced cytokine production in macrophages, I performed experiments using specific inhibitors of p38 (SB203580), p42/p44 ERK (U0126), JNK (SP600125), STAT1 (Fludarabine), STAT3 S31-201. Pre-treatment of BMDMs with SB203580, U0126, SP600125, Fludarabine and S31-201 before stimulation with *T. congolense* WCE significantly inhibited IL-6 and IL-12 production (Figure 5.2.3 A, B). Interestingly, the STAT3 inhibitor S32-201, failed to inhibit WCE-induced IL-6 and IL-12 production in macrophages, indicating that WCE-induced STAT3 signaling is not as critical as MAPKs at inducing IL-6 and IL-12 production in macrophages. Taken together, these results further confirms that the key members of MAPK (JNK, p38, ERK) and STAT1 play important role in controlling the intracellular events that lead to the production of IL-6 and IL-12 in *T. congolense* treated macrophages.

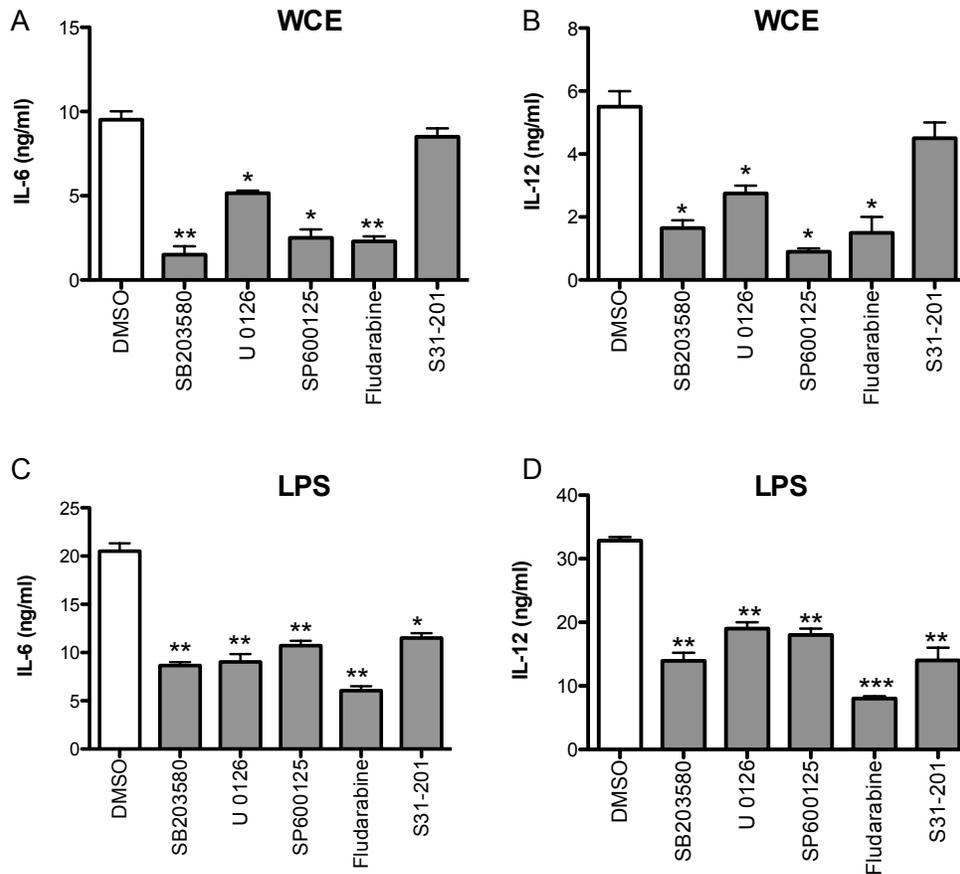


Figure 5.2.3 MAPK and STAT inhibitors abrogate the *T. congolense*-induced IL-6 and IL-12 production in macrophages.

BMDMs were incubated with MAPK inhibitors (U0126 for ERK, SB203580 for p38, SP600125 for JNK) and STAT inhibitors (Fludarabine for STAT1 and U0126 for STAT3) for 1hr. prior to inoculation with WCE (A and B) or LPS (C and D). After 24 hr., the levels of IL-6 (A and C) and IL-12 (B and D) in the culture supernatant fluids were measured by sandwich ELISA. The data presented are representative of three independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to cells treated with DMSO (vehicle).

5.2.4 *T. congolense* induces MAPK and STAT phosphorylation in peritoneal macrophages.

Next, I evaluated whether WCE-induced MAPK and STAT phosphorylation is reproducible in real infection. I injected the C57BL/6 mice intraperitoneally with WCE and assessed phosphorylation of MAPKs and STATs in the peritoneal macrophages directly *ex vivo*. As shown in Figure 5.2.4 A-C, WCE injection significantly induced phosphorylation of ERK, p38, and STAT3 in peritoneal macrophages. In another set of experiments, I injected purified live parasites into C57BL/6 mice i.p. and assessed MAPK and STAT phosphorylation in macrophages from the peritoneal lavage fluid at different times after infection. As with TC WCE, injection of live parasites also up-regulated the phosphorylation of MAPK and STATs (Figure 5.2.4 D-F), and led to a concomitant increase in IL-6 and IL-12 levels in the peritoneal lavage fluids. Collectively, these results show that *T. congolense*-induced phosphorylation of MAPK and STATs occurs following *in vivo* infection.

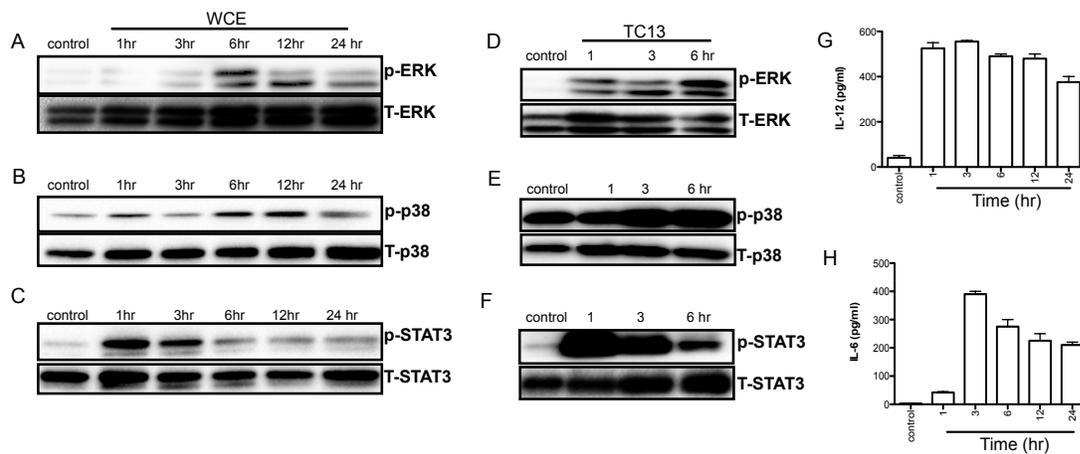


Figure 5.2.4 *T. congolense* induces MAPK and STAT phosphorylation in peritoneal macrophages.

C57BL/6 mice were inoculated intraperitoneally with 100 ml WCE containing 10^7 parasite equivalent (A-C) or 10^6 live parasites (D-F). At indicated times, the mice were sacrificed and peritoneal macrophages were isolated, pooled together, lysed and assessed directly for phosphorylation of ERK (A and D), p38 (B and E) and STAT3 (C and F) by western blot. In addition, the levels of IL-6 (G) and IL-12 (H) in the peritoneal lavage fluid of mice inoculated with WCE were also determined by ELISA. The data presented are representative of three (A-C, G and H) and two (D-F) independent experiments with similar results (n = 4-5 mice per each time point).

5.2.5 MyD88 is involved in *T. congolense*-induced intracellular signaling and cytokine production.

The adaptor molecule MyD88 and TLRs are crucial for initiating pro-inflammatory cytokine production in macrophages leading to the control of some protozoan parasites including *T. cruzi* [233, 294] and *T. brucei* [98]. However, the role of MyD88 in *T. congolense* infection has not been determined. Therefore, I inoculated BMDMs from MyD88^{-/-} mice with WCE and performed western blot to determine the MAPK and STAT phosphorylation and also assessed proinflammatory cytokine production in the culture supernatant fluids. I found that deficiency of MyD88 completely abolished WCE-induced p38 and STAT1 phosphorylation and downregulated ERK phosphorylation in macrophages (Figure 5.2.5 A-C), and this was associated with a concomitant downregulation of IL-6 and IL-12 production in the culture supernatant fluids. The results also show that MyD88 plays a crucial role in proinflammatory cytokine production in macrophages following *T. congolense* stimulation, suggesting that this adaptor molecule may be involved in the innate immune response to the parasite.

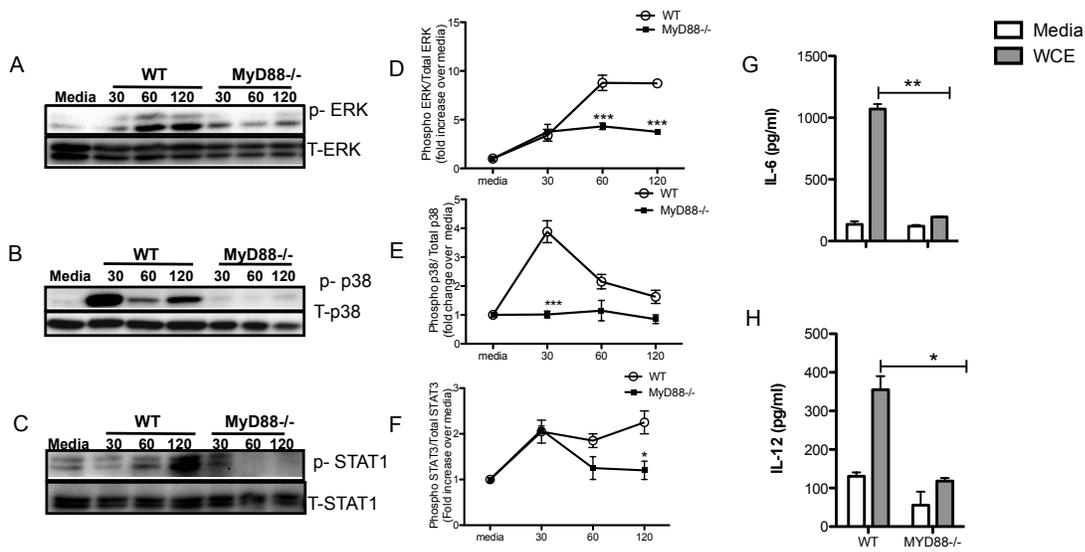


Figure 5.2.5 Activation of MyD88 is involved in *T. congolense*-induced MAPK and STAT phosphorylation and cytokine production in macrophages.

BMDMs were differentiated from marrow cells isolated from WT and MyD88^{-/-} mice and stimulated with WCE. At indicated times, the cells were lysed and lysates were assessed by western blot for phosphorylation of ERK (A and D), p38 (B and E) and STAT1 (C and F) using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38 and STAT1 and used as loading controls. The ratios of phosphorylated ERK (D), p38 (E) and STAT1 (F) to their respective total proteins were calculated by densitometry and plotted as line graphs (D-F). In addition, the levels of IL-6 (G) and IL-12 (H) in the culture supernatant fluids were determined by ELISA (G, H). The data presented are representative of two independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

5.2.6 TLR-4 is not required for WCE induced MAPK and STAT signaling and proinflammatory cytokine production.

The Toll like receptors (TLR) are important innate immune receptors involved in recognition of conserved molecular patterns expressed by microbes and the initiation of innate immune responses including inflammation. Given that activation of MyD88 is usually associated with ligation of several TLRs, I sought to determine the TLR that is involved in WCE recognition leading to the production of proinflammatory cytokines. I found that WCE-induce phosphorylation of ERK, p38 and STAT1 (Figure 5.2.6 A-C) and the production of IL-6, IL-12 and TNF production (Figure 5.2.6 J-I) were not different in TLR4^{-/-} compared to WT macrophages. As a positive control, proinflammatory cytokine production in TLR4^{-/-} macrophages was completely abolished upon LPS stimulation.

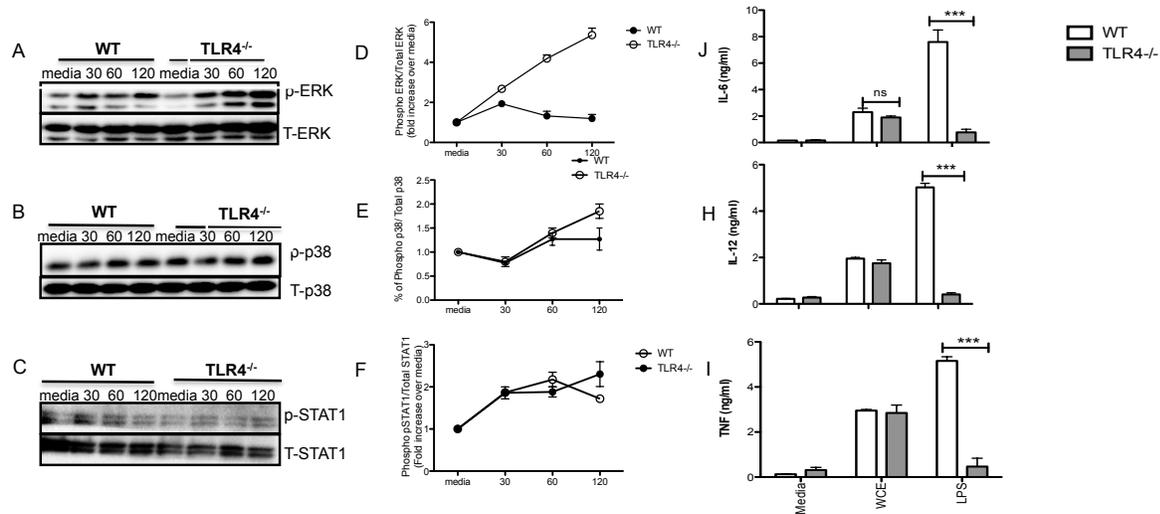


Figure 5.2.6 TLR4 is not required for *T. congolense*-induced MAPK and STAT phosphorylation and cytokine production in macrophages.

BMDMs were differentiated from WT and TLR4^{-/-} mice and stimulated with WCE. At the indicated times, the cells were lysed and lysates were assessed by western blot for phosphorylation of ERK (A), p38 (B) and STAT1 (C) using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38 and STAT1 and used as loading controls. The ratios of phosphorylated ERK (D), p38 (E) and STAT1 (F) to their respective total proteins were calculated by densitometry and plotted as line graphs (D-F). Some cells were stimulated with WCE or LPS for 24 hr. and the levels of IL-6 (G), IL-12 (H) and TNF (I) in the culture supernatant fluids were determined by ELISA. The data presented are representative of two independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. ns; not significant.

5.2.7 TLR2 is essential for WCE-induced MAPK and STAT signaling and proinflammatory cytokine production.

TLR2 has been shown to play an important role in the recognition of many parasitic infections. TLR2 expression has been shown to be essential for induction of IL-12, TNF and NO by GPI anchors derived from *T. cruzi* trypomastogotes [295]. Therefore we sought to investigate whether TLR2 has a role in *T. congolense* induced cytokine production. To investigate this I used macrophages from TLR2 deficient mice and stimulated with TC and performed western blot to see the MAPK and STAT signaling and cytokine production. In contrast to TLR4^{-/-} cells, the phosphorylation of p38, ERK, STAT1 and STA3 was abolished in the TLR2^{-/-} macrophages compared to the WT (Figure 5.2.7 A-D) and this was associated with significant reduction in IL-6, IL-12 and TNF production (Figure 5.2.7 I-K) in the culture supernatant fluids of TLR2^{-/-} compared to WT cells. Collectively, these results show that TLR2 is the innate receptor involved in *T. congolense* induced activation of MAPKs and STATs and the production of inflammatory cytokines in macrophages. This further suggests that TLR2 may play a critical role in regulating the outcome of *T. congolense* infection.

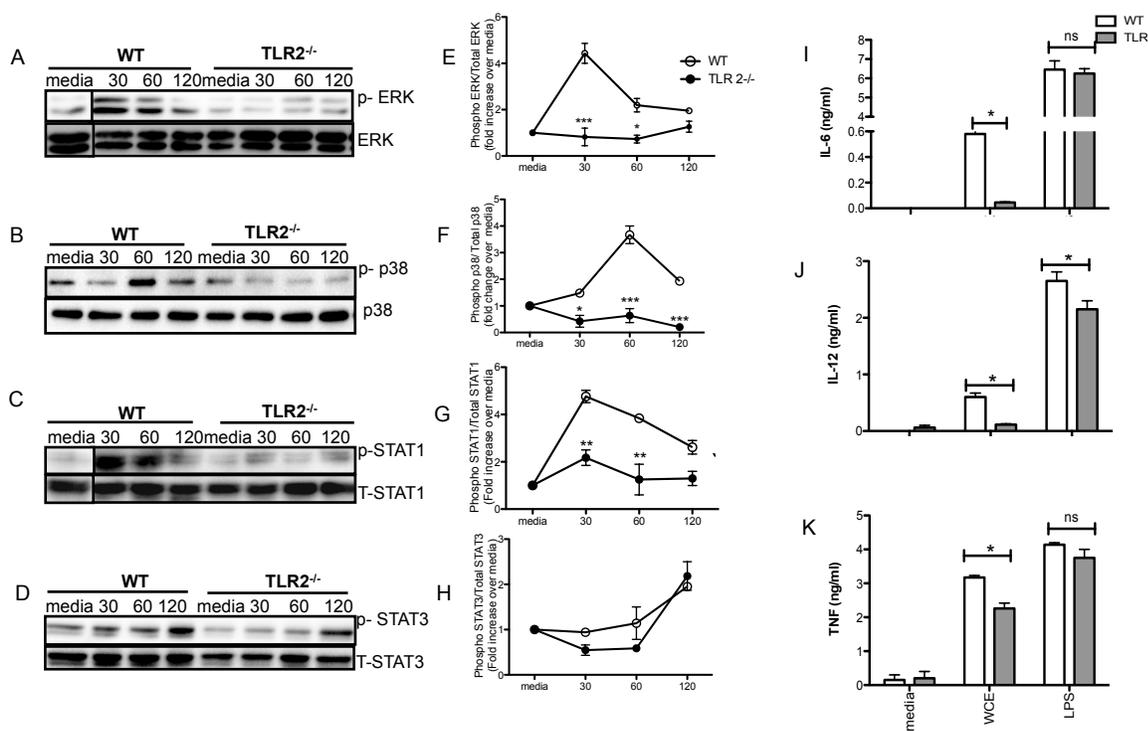


Figure 5.2.7 TLR2 expression is essential for *T. congolense*-induced MAPK and STAT phosphorylation and cytokine production in macrophages.

BMDMs were differentiated from WT and TLR2^{-/-} mice and stimulated with WCE. At the indicated times, the cells were lysed and lysates were assessed by western blot for phosphorylation of ERK (A), p38 (B), STAT1 (C) and STAT3 (D) using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38, STAT1 and STAT3 and used as loading controls. The ratios of phosphorylated ERK (E), p38 (F), STAT1 (G) and STAT3 (H) to their respective total proteins were calculated by densitometry and plotted as line graphs (E-H). Some cells were stimulated with WCE or LPS for 24 hr. and the levels of IL-6 (I), IL-12 (J) and TNF (K) in the culture supernatant fluids were determined by sandwich ELISA. The data presented are representative of two independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. ns; not significant.

5.2.8 Deficiency of TLR2 leads to uncontrolled parasitemia and acute death in the relatively resistant C57BL/6 mice

The preceding observation shows that TLR2 is essential for *T. congolense*-induced MAPK and STAT phosphorylation and proinflammatory cytokine production in macrophages, suggesting that they may play a critical role in the outcome of infection. Therefore, I infected WT and TLR2^{-/-} mice with *T. congolense* and monitored daily parasitemia and survival over time. Paradoxically, infected TLR2^{-/-} mice developed higher and uncontrolled first wave of parasitemia compared to WT mice and died within 10 days post-infection (Figure 5.2.8 A,B). In contrast, all the infected WT mice controlled several waves of parasitemia and survived up to 30 days (when the experiment was terminated). Interestingly, the acute death in TLR2^{-/-} mice was associated with significantly increased IFN- γ and TNF production in their spleens and livers compared to infected WT mice (Figure 5.2.8 C-N). Even though there was no difference in the IL-10 production between TLR2^{-/-} and WT mice, the increased IFN- γ and TNF may account for the enhanced susceptibility of these mice. The enhanced cytokine production in the liver and spleen is correlated with enhanced TNF and IFN- γ levels in the serum of the infected TLR2^{-/-} mice. Collectively, these results suggest that TLR2 is important in *T. congolense* recognition by macrophages and optimal resistance to this parasite.

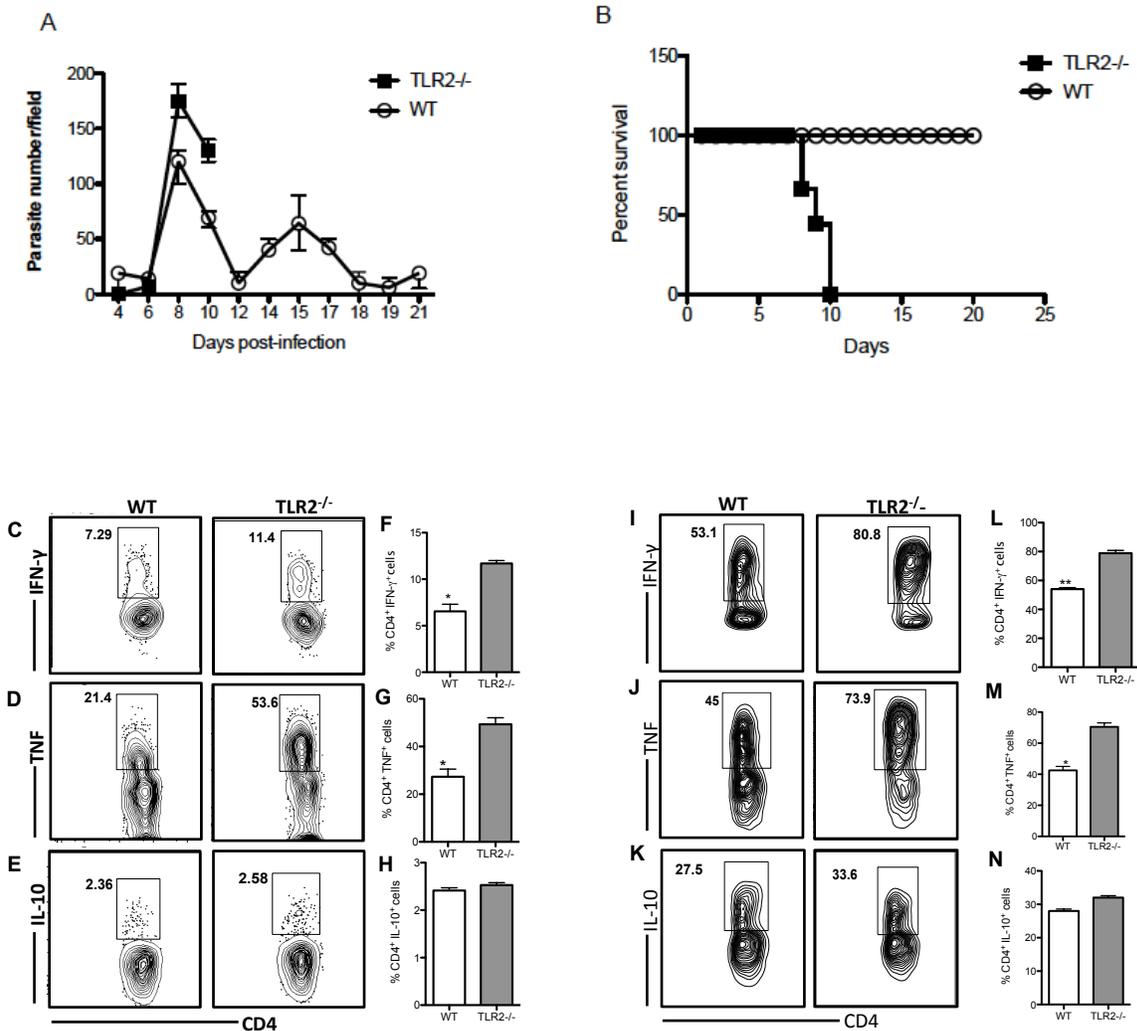


Figure 5.2.8. Deficiency of TLR2^{-/-} leads to uncontrolled first parasitemia and acute death following *T. congolense* infection.

Groups (n = 12) of TLR2^{-/-} and C57BL/6 (WT) mice were infected with 10³ *T. congolense* i.p and monitored daily for parasitemia (A) and survival (B) for about 21 days. On day 7 post-infection, the mice were sacrificed and spleen (C-E) and liver (F-H) lymphocytes were isolated and stained directly *ex vivo* for IFN- γ (C and F), TNF (D and G) and IL-10 (E and H) using appropriate fluochrome-conjugated antibodies and assessed by flow cytometry. Bar graphs represent the mean \pm SEM of the pooled results from 8-12 mice. The data presented are representative of two independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$. ns; not significant.

CHAPTER 6

6.0 Diminazene aceturate (Berenil) downregulates *Trypanosoma congolense* induced pro-inflammatory cytokine production by altering the phosphorylation of MAPK and STAT proteins.

6.1. Introduction

African Trypanosomes are extracellular protozoan parasites that cause fatal disease in both humans and animals. These parasites never enter the host cells, but can survive for extended period of time in mammalian blood and tissues. Although the disease kill hundreds of thousands of people in underdeveloped regions, current treatment strategies are not satisfactory. There are only few drugs available and these drugs have several limitations and are ineffective. According to WHO reports Human African trypanosomiasis (HAT) is a major threat to the health of 60 million people in 36 countries in Sub-Saharan Africa. Animal trypanosomiasis adversely affects the live stock production and farming in the affected areas. *T. congolense* is one of the most important pathogens for livestock and thus contribute significantly to food and economic security. It is estimated that the annual production loss of cattle is about \$12,000 million in Sub-Saharan African countries [6].

In the mammalian host African trypanosomes are characterized by a unique immunogenic surface coat of variant surface glycoprotein (VSG) that is anchored to the membrane by Glycosyl Phosphatidyl Inositol (GPI). These parasites escape the host immune response by constantly modifying their VSG by a process of antigenic variation causing the fluctuating waves of parasitemia and this is a characteristic feature of African Trypanosomes [15, 296]. The antigenic variation is the central most important evasion mechanisms that the parasite has evolved to survive in the chronically infected host [297]. The other evasion mechanisms are

polyclonal B-lymphocyte activation [290], marked immunosuppression [250, 290] and all these together accounts for the failure to design an effective vaccine .

Macrophages are the key players of innate immune system in microbial infections. Macrophages sample the extracellular environment for foreign molecules by receptor mediated detection and internalization events. Ligation of Toll-like receptors by molecular signatures of microbial infection, initiates signaling cascades that result in macrophage activation and production of inflammatory factors that amplify the innate immune response to infection and stimulate adaptive immunity [298]. It has been shown that macrophage activation can be detected within few days of infection with African Trypanosomes. Activated macrophages enhance gene transcription leading to the release of inflammatory mediators including cytokines TNF, IL-6, IL-12 and NO production following infection with Trypanosoma.

Berenil otherwise called as diminazene aceturate has been used for the treatment of animal trypanosomiasis for over 60 years. Berenil is the most commonly used therapeutic agent for trypanosomiasis in livestock. Despite of its use for almost 60 years, Berenil's mechanism of action is poorly understood. A complete understanding of the molecular mechanism of action is critical in order to maximize the therapeutic and prophylactic potentials. In the preceding chapters, I showed that Berenil modulates host immune response to the parasite. Berenil treatment down-regulates pro-inflammatory cytokines in serum and alters the activation status of lymphocytes in spleens and livers of infected mice. I also showed that Berenil treatment down-regulates LPS induced pro-inflammatory cytokine production in macrophages. In addition, I identified the Toll like receptor involved in *T. congolense* infection by using both *in vitro* and *in vivo* approaches and investigated the adaptor proteins and important signaling pathways involved in *T. congolense*-induced cytokine production. I showed that ERK, p38,

STAT1 and STAT3 are involved in the *T. congolense* mediated IL-6, IL-12 and TNF production. In this part of the thesis, I investigated whether Berenil pre-treatment has any effect on *T. congolense*-activated signaling pathways in macrophages.

6.2 RESULTS

6.2.1 Berenil pre-treatment down-regulates *T. congolense* (TC13)- induced IL-6 and IL-12 production in macrophage cell lines (ANA) and BMDM.

I previously showed that Berenil treatment dramatically lowers the serum pro-inflammatory cytokine production in *T. congolense* infected mice. I also found that this was due to the direct effect on CD11b⁺ macrophages from spleen and liver in Berenil treated mice. I also tested the cytokine production from macrophage cell lines (ANA-1) and BMDMs in response to inoculation with *T. congolense in vitro* and I found that there was an up-regulation of IL-6 and IL-12. To more clearly determine whether Berenil pre-treatment has any effect on this *T. congolense* induced cytokine production we pre-treated immortalized macrophage cell lines and bone marrow derived macrophages with Berenil for ON and then stimulated with *T. congolense* lysate for 12- 18 hrs. Berenil treatment suppressed *T. congolense* induced IL-6 and IL-12 production in both macrophage cell lines and primary macrophages (Figure 6.2.1 A-D). This result indicates that as observed *in vivo*, Berenil pretreatment down-regulates the *T. congolense* induced cytokine production in macrophages *in vitro*.

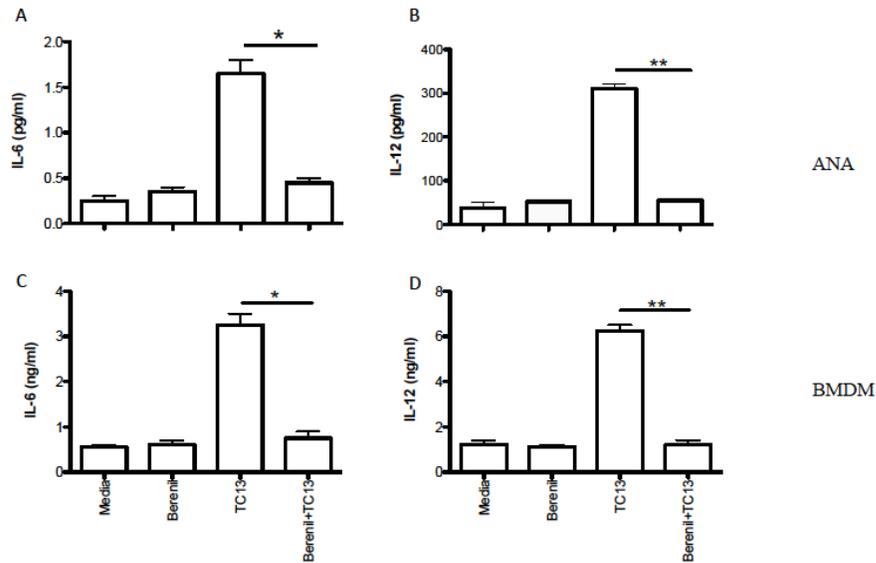


Figure 6.2.1 Berenil pre-treatment down-regulates *Trypanosoma congolense* (TC13) induced cytokine production in macrophage cell lines (ANA) and bone marrow derived macrophages (BMDM).

ANA cells and BMDM were treated with Berenil (10 $\mu\text{g/ml}$) overnight and inoculated with TC13 (1:10 ratio). After 14-16 h, the culture supernatant fluids were collected and assayed for IL-6 and IL-12 production by sandwich ELISA. Shown are IL-6 and IL-12 production by ANA (A,B) and BMDM (C,D). The data presented are representative of three different experiments with similar results. * $p < 0.05$ ** $p < 0.01$.

6.2.2. Berenil pretreatment downregulates TC13-induced MAPK and STAT phosphorylation in ANA cells.

MAPK and STATs are important signaling molecules that regulate pro-inflammatory cytokine production in immune cells [299]. The death of *T. congolense* infected mice is partly due to the enhanced cytokine production leading to systemic inflammatory response like syndrome. Several parasites have been shown to activate the MAPKs signaling pathways during infection. *Trypanosoma cruzi* and *Toxoplasma gondii* have shown to increase the phosphorylation of ERK, p38 and JNK [210, 293]. I recently identified the molecular mechanisms underlying the cytokine production in response to *T. congolense* in macrophages. I found that MAPKs, STAT1 and STAT3 phosphorylation is up regulated in response to *T. congolense* infection. In addition, I previously showed that Berenil treatment down regulates the pro-inflammatory cytokine production in *T. congolense* infected mice. Therefore I wanted to investigate whether Berenil pre-treatment has any effect on TC-induced MAPK, STAT1 and STAT3 phosphorylation. To test this, I pretreated the ANA cells with Berenil, inoculated with TC13 and collected the lysate at various time points to perform western blot. Interestingly, I found that pre-treatment with Berenil did not alter ERK phosphorylation, but inhibited the p38, STAT1 and STAT3 phosphorylation in ANA cells (Figure 6.2.2 A-D) suggesting that the inhibitory effect of Berenil on cytokine production is mediated by down regulation of p38, STAT1 and STA3 pathways and the inhibitory effect of Berenil is not global.

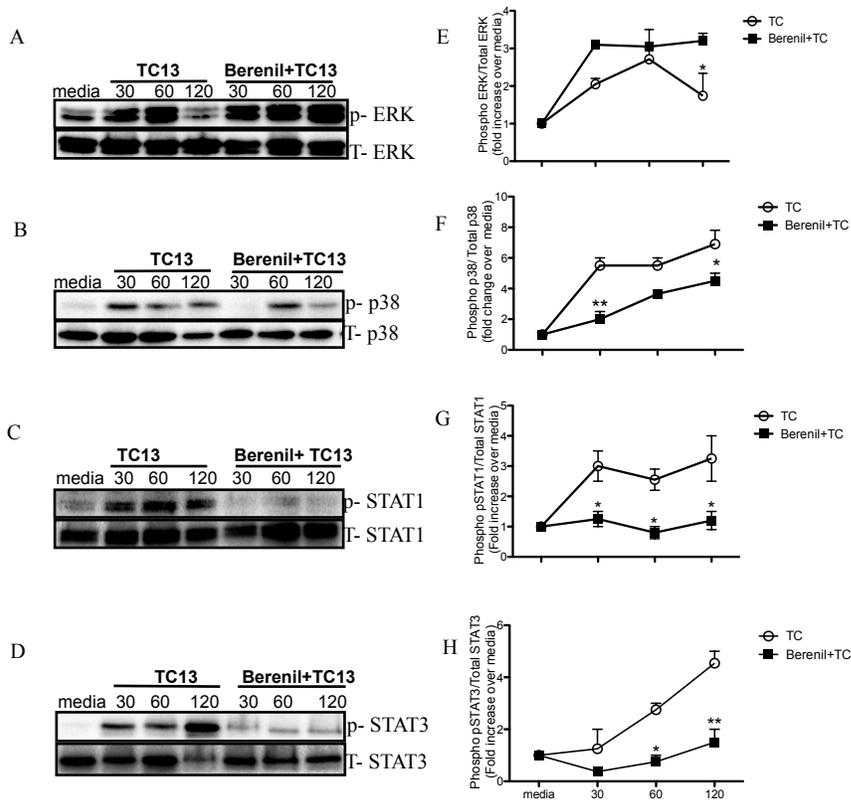


Figure 6.2.2 Berenil alters TC13 induced MAPK and STAT phosphorylation in ANA cells.

ANA cells were treated with Berenil (10 $\mu\text{g/ml}$) overnight and inoculated with TC13. At indicated times the cells were lysed and the lysates were assessed by Western blot for phosphorylation of ERK (A), p38 (B), STAT1 (C) and STAT3 (D). The same blots were stripped and re-probed with Abs against total ERK, p38, STAT1, STAT3 and used as loading controls. The right panel shows the corresponding densitometry (E-H). The western blot results represent one of the three independent experiments with similar findings.

6.2.3 Berenil pretreatment down-regulates TC13 induced MAPK and STAT phosphorylation in bone marrow derived macrophages (BMDMs).

I found that Berenil treatment alters *T. congolense*-induced MAPK and STAT phosphorylation in macrophage cell lines. Next, I tested whether the effect of Berenil on MAPK and STAT phosphorylation is reproduced in a primary macrophage, BMDMs. Therefore I pretreated the BMDMs with Berenil and stimulated with TC13 and performed western blot from lysates to see the effect of Berenil on MAPK and STAT phosphorylation. In line with ANA cells Berenil treatment did not alter the ERK phosphorylation but down regulated the p38, STAT1 and STAT3 phosphorylation (Figure 6.2.3 A-D). These results indicate that Berenil has an effect on inhibiting the cytokine production and this inhibition is mediated by down regulating the phosphorylation of p38, STAT1 and STAT3 that are important signaling molecules in inflammation.

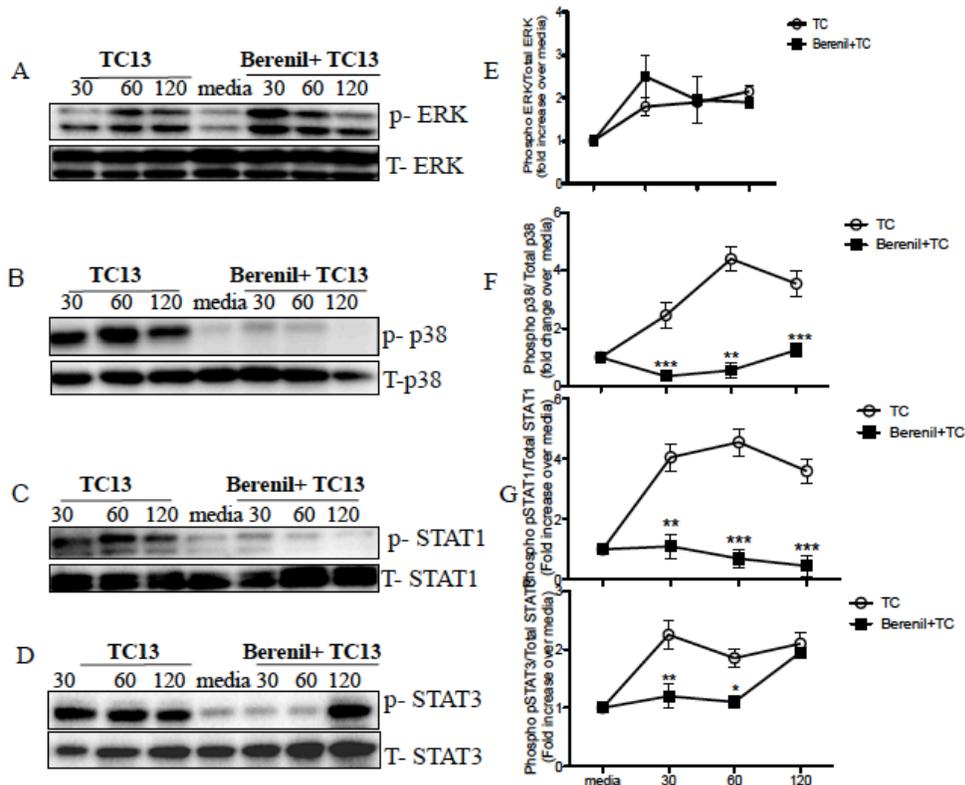


Figure 6.2.3. Berenil down-regulates TC13 induced MAPK and STAT phosphorylation in BMDM.

Macrophages were treated with Berenil (10 $\mu\text{g/ml}$) overnight and inoculated with TC13. At indicated times the cells were lysed and the lysates were assessed by Western blot for phosphorylation of ERK (A), p38 (B), STAT1 (C) and STAT3 (D). The same blots were stripped and re-probed with Abs against total ERK, p38, STAT1, STAT3 and used as loading controls. The western blot results represent one of the three independent experiments with similar findings.

6.2.4 Berenil pretreatment down-regulates TC13 induced MAPK and STAT phosphorylation in peritoneal macrophages

Next, I evaluated whether the effect of Berenil on TC induced MAPK and STAT phosphorylation is reproducible in real infection. I pre-treated the mice with Berenil (14 mg/kg) and after 14-18 hr, injected them with TC13 lysate, sacrificed them at different time points and assessed the phosphorylation of ERK, p38, STAT1 and STAT3 in peritoneal macrophages directly *ex vivo*. Berenil treatment significantly down regulated TC13-induced phosphorylation of p38, STAT1 and STAT3, but did not affect the ERK phosphorylation (Figure 6.2.4 A-C). Consistent with this, Berenil treatment caused significant reduction in IL-6 and IL-12 levels in the peritoneal lavage fluids of TC treated mice (Figure 4 E, F). Collectively, these results further confirm that Berenil downregulates TC- induced pro-inflammatory cytokine production by altering the phosphorylation of MAPKs and STATs *in vivo*.

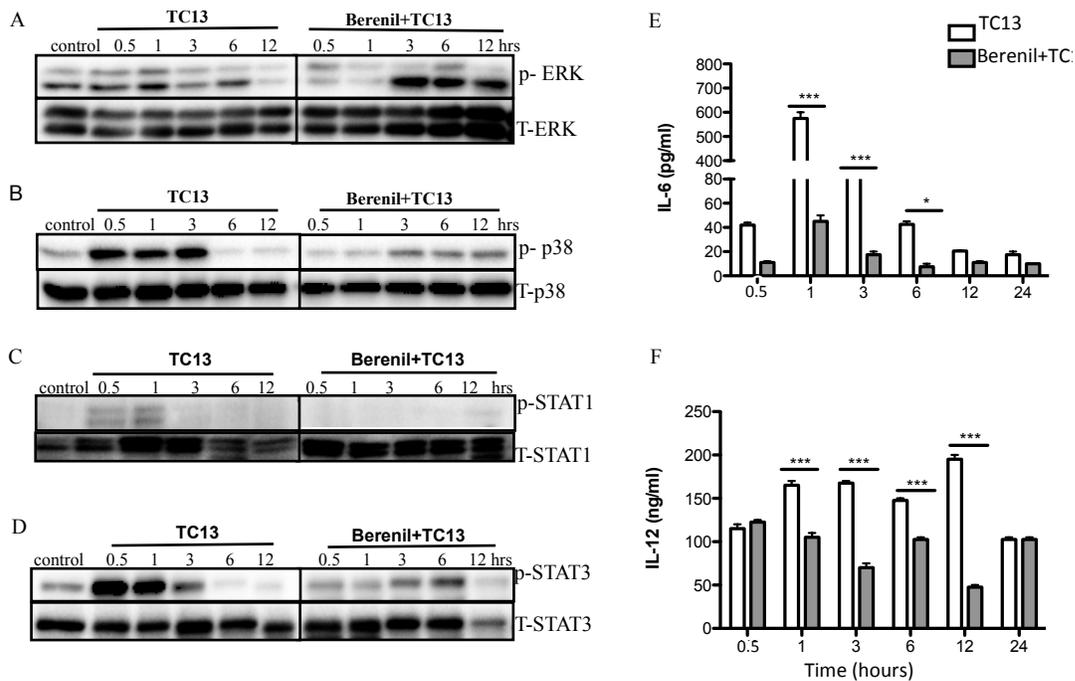


Figure 6.2.4. Berenil pretreatment down-regulates *T. congolense* induced MAPK and STAT phosphorylation in peritoneal macrophages.

Groups of C57BL/6 mice were treated with Berenil ON and then inoculated with TC13 (10^8 parasites/ml). At indicated times, mice were sacrificed and lysates of peritoneal macrophages were assessed directly for phosphorylation of ERK, p38, STAT1 and STAT3 (A-D) by western blot. The data presented are representative of two independent experiments with similar results (n=2 mice per each time point). In addition, the IL-6 and IL-12 levels in the supernatants were measured by ELISA (E, F). The data presented are representative of two independent experiments with similar results (n=2 mice per each time point). * $p < 0.05$ *** $p < 0.001$

6.2.5 Berenil down-regulates TC induced phosphorylation of NFκB p65 and IL-6 promoter activity.

Nuclear factor-κB (NFκB) family of transcription factors plays critical roles in inflammation, cell proliferation and survival. The most activated form of NFκB is a heterodimer consisting of p65 and p50 subunit and they control genes regulating a broad range of biological processes including inflammation, stress responses etc. The activated NFκB is further translocated into the nucleus and either alone or in combination with other transcription factors induces target gene expression. In order to evaluate whether the suppression of TC induced pro-inflammatory cytokine production is dependent on NFκB activation and thus gene transcription, I performed western blot and ELISA to detect NFκB p65 phosphorylation. Pre-treatment of BMDMs with Berenil inhibited TC induced phosphorylation of NFκB p65 subunit as assessed by Western blot and ELISA (Figure 6.2.5 A, B). In order to investigate whether Berenil directly affects the promoter activity, ANA cells were transiently transfected with WT IL-6 luciferase constructs, pretreated ON with Berenil and then stimulated with TC. ANA cells transfected with IL-6 promoter construct showed increase in promoter activity in response to TC (Figure 6.2.5C)

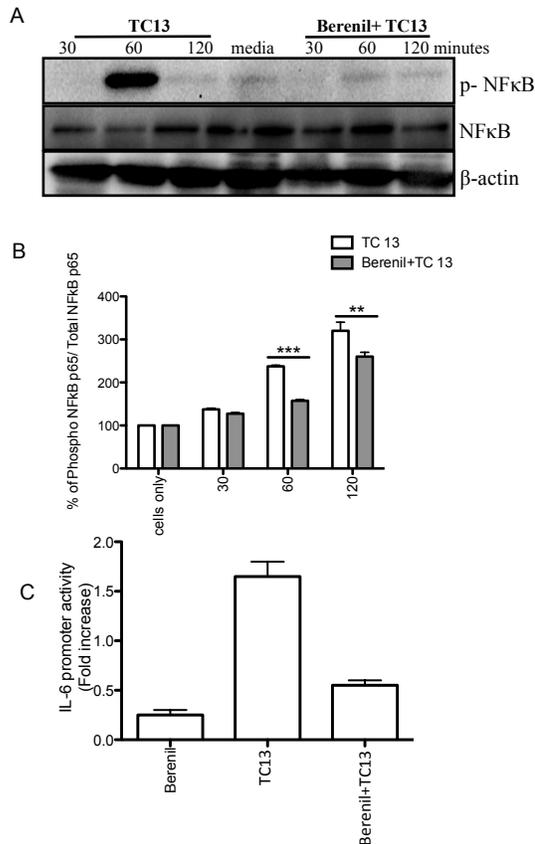


Figure 6.2.5. Berenil pretreatment downregulates TC13 induced phosphorylation of NFκB p65 subunit and IL-6 promoter activity in macrophages.

BMDM were treated with Berenil (10 μg/ml) overnight and then stimulated with TC13. At indicated times, the cells were lysed and the lysates were assessed by western blot (A) or ELISA (B) for phosphorylation of NFκB p65 subunit. The same blot was stripped and reprobbed with antibody against total NFκB and used as loading control (A). The data presented are representative of two independent experiments with similar results, ** $p < 0.01$

Macrophages were transiently transfected with IL-6 promoter construct containing luciferase gene and then pretreated with Berenil (10 μg/ml) for 12 hr. Thereafter, the cells were inoculated with TC13 for another 12 h and the luciferase reporter activity was measured. Data are presented, as fold increase over unstimulated controls and are representative of 2 independent experiments with similar findings (C).

CHAPTER 7

7.0 Diminazene aceturate (Berenil) modulates LPS induced proinflammatory cytokine production by inhibiting phosphorylation of MAPKs and STAT proteins.

7.1 Introduction

Inflammation is the earliest response of the host defense system to injury or insult including infection. During infection, an inflammatory response may be beneficial or detrimental. Inflammation is beneficial to the host if it provides protection against infection. However, uncontrolled and dysregulated inflammation can be detrimental as is the case in septic shock [300]. Thus, inflammatory process must be tightly regulated by highly coordinated signals that initiate, maintain and ultimately shut down the cascade when the insult is eliminated. Cellular and tissue damage occur when there is an imbalance that results in excessive inflammation. In addition, prolonged inflammation results in a chronic inflammatory state, which is the major pathogenic mechanism in a number of chronic diseases including rheumatoid arthritis, Inflammatory bowel disease, psoriasis etc. [301].

Toll-like receptors (TLRs) are a family of evolutionarily conserved receptors that play a crucial role in early host defense against microbial pathogens [221]. They are major pathogen recognition receptors present on macrophages and other innate immune cells that act as the first line of defense against pathogens. Upon recognition of their respective ligands, which are molecular patterns expressed on pathogens, a cascade of intracellular signaling events are activated leading to the activation of NF κ B that ultimately results in the transcription of proinflammatory genes and the production of inflammatory cytokines [302].

An inflammatory response is orchestrated by the interaction between several inflammatory mediators and immune cells [303]. Macrophages play a critical role in inflammation via antigen presentation, phagocytosis and production of proinflammatory cytokines (such as IL-6,

IL-12, TNF etc.) and other factors that result in immunomodulation [304]. Additionally, macrophages also respond to cytokines, free oxygen radicals and complement products produced as result of an inflammatory process [305]. The production and autocrine effects of inflammatory cytokines on macrophages results in the activation of transcription factors (e.g. NFκB) and protein kinases (e.g. MAP kinases), which in turn regulate the expression of many target genes including those that are involved in inflammatory cytokine production [306].

Diminazene aceturate (Berenil) has been the drug of choice for treatment of animal trypanosomiasis since 1955. It is an aromatic diamidine consisting of two amidinophenyl moieties linked by a triazene bridge [243]. In addition to its trypanocidal activity, Berenil also has excellent antibabesial activity, which has increased its field applicability [307]. Although the compound has been in use for more than half a century, the exact mechanism of action and its effect on the host immune system are still poorly understood. Some studies suggest that the compound binds to trypanosomal kinetoplast DNA (kDNA) in a non-intercalative manner through specific interaction with sites rich in adenine-thymine base pairs [245]. This causes an irreversible damage resulting in the development of akinetoplast parasites [246, 247]. Additionally, Berenil induces condensation of heterochromatin during the G2 phase of cell cycle and this causes the DNA to become completely unfolded [248]. Berenil also alters the confirmation of DNA by interfering with binding of DNA topoisomerases [249].

Although many of the anti-parasitic effects of Berenil have been attributed to its biochemical properties enumerated above, few reports suggest that the compound may have some effect on the host immune system. In *T. congolense*-infected cattle, Berenil treatment at the time of vaccination abolishes immunosuppression and enhances the immune response to *Leptospira biflexa* [250]. Treatment of *T. congolense*-infected highly susceptible BALB/c mice with

Berenil leads to control of parasitemia and a dramatic increase in plasma levels of parasite-specific IgG2a and IgG3 antibodies [74]. In addition, Berenil treatment leads to complete cure of infected mice and results in resistance to homologous challenge [251]. In a seminal study, Arowolo *et al* showed that in addition to its trypanocidal action, Berenil treatment blocks histamine-induced responses in tissues and exert some anti-histaminic and anti-inflammatory effects *in vivo* [252]. Previously, I showed that Berenil treatment of *T. congolense*-infected mice dampens T cell and macrophage hyperactivation and lowers serum levels of proinflammatory cytokines by directly altering the production of these cytokines by splenic and hepatic macrophages. Collectively, these results indicate that in addition to its trypanolytic effect, Berenil also modulates the host immune system.

In this section of the thesis, I used the well-characterized LPS challenge *in vitro* and *in vivo* studies to more clearly investigate how Berenil downregulates proinflammatory cytokine production in macrophages. I show that Berenil does not mediate its anti-inflammatory properties by altering TLR expression on macrophages. Rather, the compound strongly downregulated phosphorylation of mitogen activated protein kinases (ERK, p38 and JNK), signal transducers and activators of transcription (STAT1 and STAT3), NFκB p65 subunit and directly interferes with IL-6 promoter activity. In addition, Berenil also upregulates the expression of SOCS in macrophages, suggesting that it could exert both direct and indirect effects on proinflammatory cytokine production in macrophages. The results obtained from this section clearly and unequivocally show that the anti-inflammatory property of Berenil is not trypanosome-specific, suggesting that this old compound could be used as a non-specific agent from treating disease conditions mediated by excessive inflammatory response.

7.2 RESULTS

7.2.1 Berenil downregulates LPS-induced proinflammatory cytokines production in bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC).

I previously showed that injection of Berenil dramatically lowers serum levels of proinflammatory cytokines (including IL-6, IL-12p40 and TNF) in mice infected with *T. congolense* or following LPS challenge. This effect was mediated by direct modulatory effect on splenic and hepatic CD11b⁺ cells from treated mice. To more clearly determine the molecular mechanisms through which Berenil suppresses proinflammatory cytokines production, I pretreated immortalized macrophage cell line (ANA-1) or primary BMDMs and BMDCs overnight with varying concentrations of the compound (ranging from 10 ng/ml to 10 µg/ml) and then stimulated them with LPS (1 µg/ml) for 12-16 hrs. Berenil treatment suppressed LPS-induced IL-6, IL-12p40 and TNF production in a dose-dependent manner in both immortalized cell line (Appendix 2A-C) and primary BMDMs. (Figure 7.2.1 A-C).

To determine whether the effect of Berenil was restricted to macrophages only, I also assessed LPS induced proinflammatory cytokine production by BMDC following pretreatment with Berenil. As shown in Figure 7.2.1 D-F and consistent with the effects observed in BMDMs, Berenil also downregulated LPS induced proinflammatory cytokine production by BMDCs, suggesting that the effect is not restricted only to macrophages. Interestingly, Berenil did not downregulate the production of MCP-1 by macrophages (Appendix 3), suggesting that it does not exhibit global inhibitory effect on inflammatory cytokine release. In addition, at less than 10 µg/ml, the drug was non-toxic to the cells as assessed by MTT, trypan blue dye exclusion and propidium iodide assays (Appendix 4A-C). These results indicate that as

observed *in vivo* in chapter IV, Berenil directly and significantly reduced the production of proinflammatory cytokines by macrophages and dendritic cells *in vitro*.

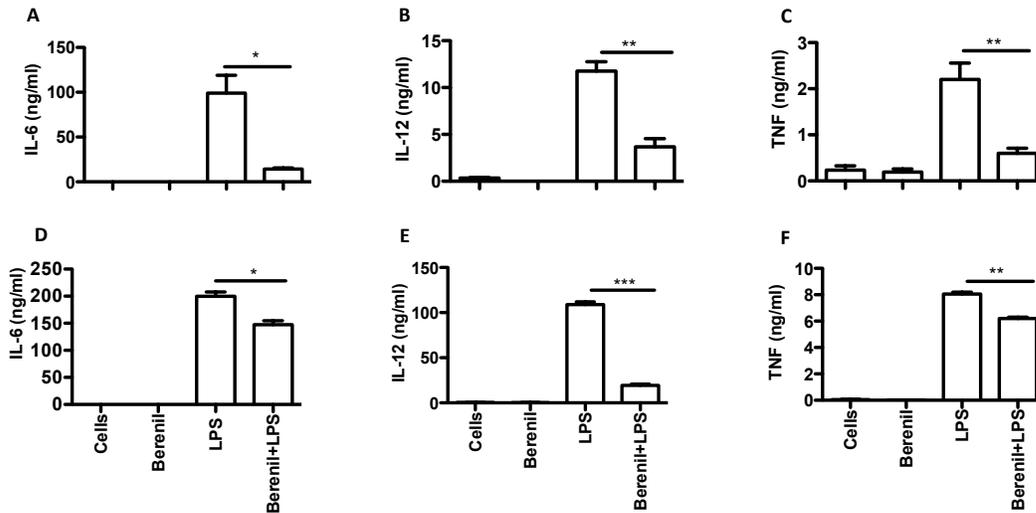


Figure 7.2.1. Berenil pre-treatment downregulates LPS-induced cytokine production in macrophages and dendritic cells. Bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) were differentiated from marrow cells isolated from C57BL/6 mice, treated with Berenil (10 $\mu\text{g/ml}$) overnight and stimulated with LPS (1 $\mu\text{g/ml}$). After 12 hr, the culture supernatant fluids were collected and assayed for IL-6 (A and D), IL-12 (B and E) and TNF (C and F) by sandwich ELISA. Shown are IL-6, IL-12 and TNF production by BMDMs (A-C) and BMDCs (D-F). The data presented are representative of 3 independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

7.2.2 Berenil downregulates CpG induced-production of proinflammatory cytokines in macrophages and dendritic cells.

Next, I assessed whether the downregulatory effects of Berenil on proinflammatory cytokine production was global or specific to LPS stimulation. Bacterial DNA and synthetic oligonucleotides (ODN) containing unmethylated CpG motifs (CpG-ODN) can stimulate cells of innate and adaptive immune system (including macrophages) leading to increased proinflammatory cytokine production [308-310]. Because CpG DNA receptor (Toll like receptor (TLR 9) shares common signaling pathways with the LPS receptor (TLR4) [311], I compared the cytokine production in Berenil pretreated BMDM and BMDC following CpG ODN stimulation. Pre-treatment of BMDMs and BMDCs with Berenil significantly downregulated CpG-induced IL-6, IL-12p40 and TNF production (Figure 7.2.2 A-F). In addition, Berenil treatment also downregulated Poly I:C (TLR3 agonist) induced IL-6 production by BMDMs (Appendix 5).

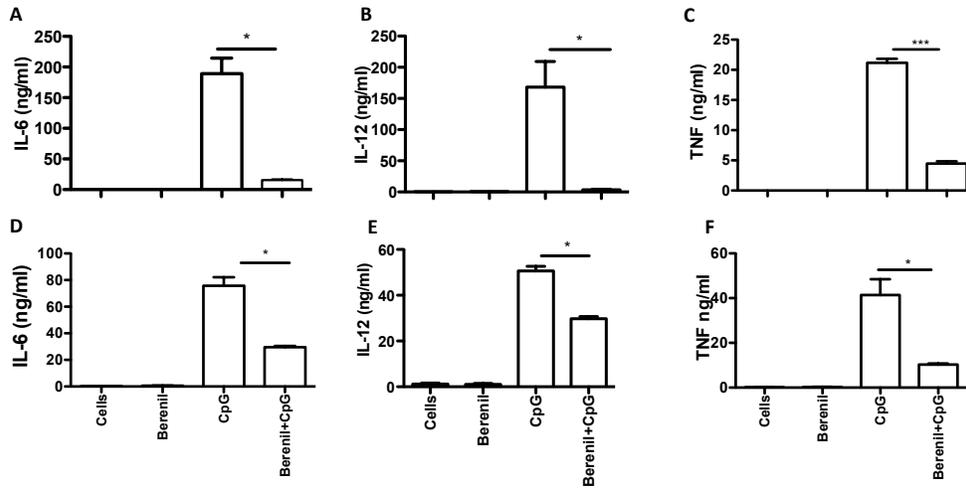


Figure 7.2.2 Berenil pre-treatment downregulates CpG-induced cytokine production in macrophages and dendritic cells. BMDMs and BMDCs were treated with Berenil (10 μ g/ml) overnight, stimulated with CpG ODN (5 μ g/ml) for additional 12 hr and the culture supernatant fluids were assayed for IL-6 (A and D), IL-12 (B and E) and TNF (C and F) by sandwich ELISA. Shown are IL-6, IL-12 and TNF production by BMDMs (A-C) and BMDCs (D-E). The data presented are representative of 3 independent experiments with similar findings. *, p < 0.05; ***, p < 0.001.

7.2.3 Berenil does not alter the Toll Like Receptor expression on macrophages

TLRs are innate immune sensors that recognize pathogen-associated molecular patterns (PAMPs). Binding of specific ligands/agonists to their respective TLRs activates cell signaling pathways leading to up-regulation of costimulatory molecules and the production of proinflammatory cytokines [312]. Because I found that pre-treatment of BMDMs with Berenil significantly suppressed their production of IL-6, IL-12p40 and TNF, I hypothesized that Berenil alters TLR expression on macrophages. Therefore, I pre-treated BMDMs with varying concentrations of Berenil (0.01-10 $\mu\text{g/ml}$) and assessed the expression of TLR4-MD2 complex and CD14, which is involved in LPS recognition and signaling [313]. I found that pre-treatment with Berenil did not alter the surface expression of TLR4-MD2 complex and CD14 on macrophages (Figure 7.2.3 A-D and data not shown). Some reports show that signaling via TLR2 is required along with TLR4 for optimal LPS-induced intracellular signaling that leads to proinflammatory cytokines production in macrophages [314]. Therefore, I considered the possibility that Berenil might be altering the TLR2 expression. As seen for TLR4, Berenil pre-treatment did not affect the TLR2 expression (Figure 7.2.3 E and F). Similarly, Berenil pre-treatment did not alter TLR9 expression on macrophages (Figure 7.2.3 G and H). Collectively, these results show that the downregulation of proinflammatory cytokines production by Berenil is not due to alteration in TLR expression on macrophages, suggesting that it may be altering downstream intracellular signaling pathways following ligation of TLRs by their respective ligands.

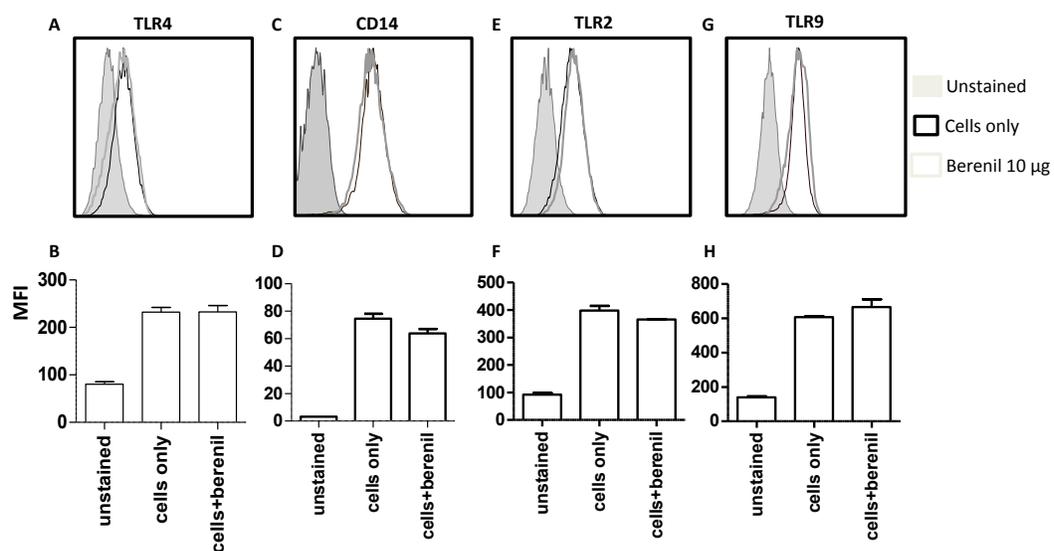


Figure 7.2.3 Berenil treatment does not affect the expression of toll-like receptors and other key LPS binding molecule on macrophages. BMDM were treated with or without Berenil (10 µg/ml) overnight, stained with PE-labelled monoclonal antibody (mAb) against TLR4 and TLR2, APC-conjugated mAb against CD14 and FITC-conjugated mAb against TLR9 and analyzed by flow cytometer. Shown are representative histogram of TLR4, CD14, TLR2 and TLR9 (A, C, E and G, respectively). The bar graphs represent the corresponding mean fluorescence intensities (MFIs, B, D, F and H). The data presented are representative of 3 independent experiments with similar findings.

7.2.4 Berenil pre-treatment downregulates LPS-induced phosphorylation of MAP

Kinases and STATs in macrophages

Mitogen activated protein kinases (MAPKs) and signal transducer and activator of transcription (STAT) are critical signaling molecules that regulate proinflammatory cytokine production in immune cells [299, 315]. Since I found that Berenil does not mediate its inhibitory effects on proinflammatory cytokines production by altering TLR expression and given the fact that I previously found that Berenil inhibits *T. congolense*-induced MAPK and STAT activation *in vivo* and *in vitro* (see chapter VI), I speculated that the drug might be altering these intracellular signaling molecules and pathways that regulate proinflammatory cytokine gene expression in macrophages. Therefore, I used western blot to investigate the effect of Berenil pre-treatment on phosphorylation of BMDMs *in vitro*. Pre-treatment with Berenil inhibits LPS-induced phosphorylation of ERK, JNK and P38, (Figure 7.2.4 A-D) and STATs (STAT1 and STAT3; Figure 7.2.5 A-D). Interestingly, pre-treatment with Berenil did not suppress, but rather enhanced STAT5 phosphorylation (Figure 7.2.5E and F), suggesting the compound does not globally affect intracellular phosphorylation events in macrophages. Collectively these results suggest that the inhibitory effect of Berenil on cytokine production is mediated by the downregulation of MAPKs and STATs signaling.

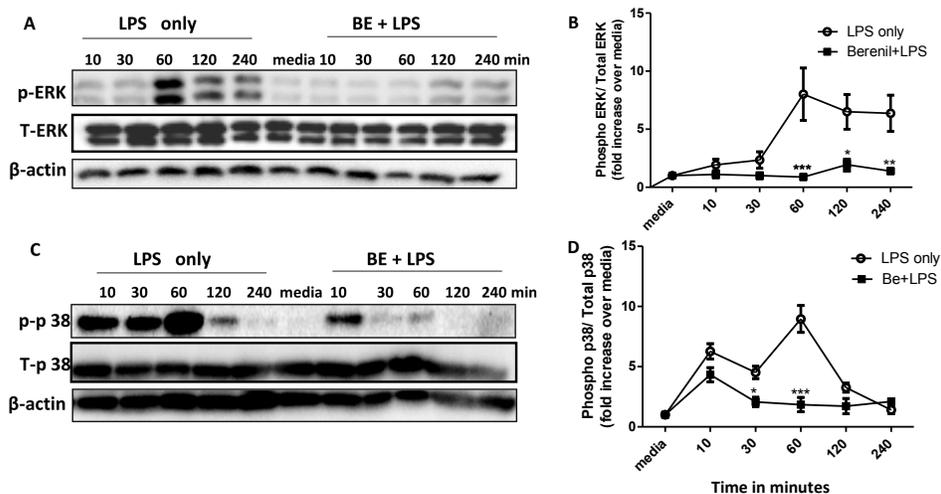


Figure 7.2.4. Berenil downregulates the phosphorylation of MAPKs in macrophages. BMDM were treated with Berenil (10 $\mu\text{g/ml}$) overnight and then stimulated with LPS. At indicated times, the cells were lysed and the lysates were assessed by western blot for phosphorylation of ERK and p38, (A-D). The same blots were stripped and reprobbed with antibodies against total ERK and p38 and used as loading controls. β -actin was also done as loading control. The western blot results represent one of three independent experiments. Densitometric analysis was performed from three blots and presented as ratio of phosphorylated proteins over total (unphosphorylated) controls compared to media. The data presented are representative of three independent experiments with similar findings. *, $P < 0.05$; ***, $p < 0.001$.

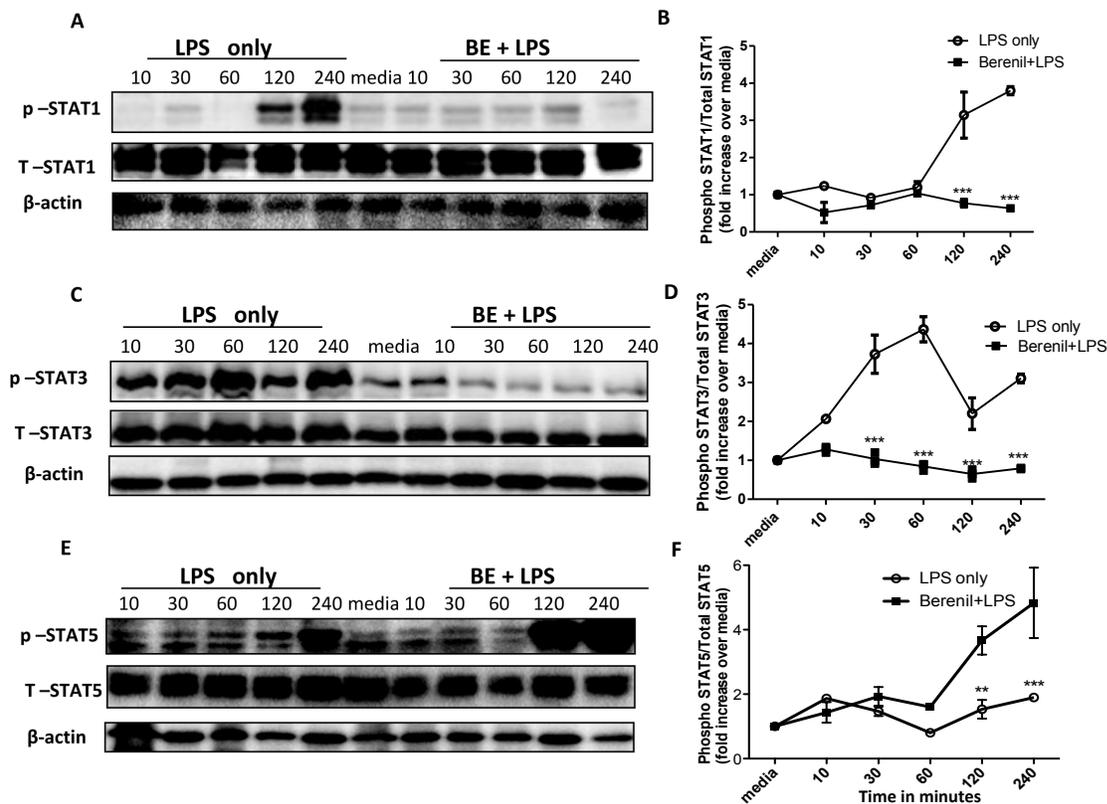


Figure 7.2.5. Berenil down-regulates the phosphorylation of STATs in macrophages. BMDM were treated with Berenil (10 μ g/ml) overnight and then stimulated with LPS (1 μ g/ml). At indicated times, cells were lysed and the lysates were assessed by western blot for phosphorylation of STAT1, STAT3 and STAT 5 (A-F). The same blots were stripped and reprobbed with antibodies against total STAT1, STAT3 and STAT5 and used as loading control. The western blot results represent one of three independent experiments. Densitometric analysis was performed from three blots and presented as ratio of phosphorylated proteins over total (unphosphorylated) controls and compared to the media control. The data presented are representative of three independent experiments with similar findings. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

7.2.6 Berenil downregulates the LPS-induced phosphorylation of MAPKs and STATs in peritoneal macrophages

Next, I evaluated whether Berenil-induced downregulation of LPS-induced MAPKs and STAT phosphorylation was reproducible *in vivo*. I pre-treated mice with Berenil (14 mg/kg), and after 16-24 hrs, isolated the peritoneal macrophages and restimulated them *in vitro* with LPS (1µg/ml). Consistent with our *in vitro* observations, treatment with Berenil downregulated LPS-induced phosphorylation of ERK, p38, STAT1 and STAT3. The p38 and STAT3 phosphorylation is shown in (Figure 7.2.6A and B) at different times tested.

To further confirm the effect of Berenil on MAPK and STAT phosphorylation in a more relevant *in vivo* setting, I pre-treated mice with Berenil for 24 hr, injected them with LPS (5 mg/kg) and at different times, sacrificed them and assessed phosphorylation of MAPKs and STATs in the peritoneal macrophages directly *ex vivo*. Berenil treatment significantly downregulated LPS-induced phosphorylation of p38 and STAT3 (Figure 7.2.6 C and D). Consistent with this, Berenil treatment caused significant reduction in the levels of IL-6 and TNF in the peritoneal lavage fluids of LPS treated mice (Figure 7.2.6 E and F). Collectively, these results further confirm that Berenil downregulates LPS-induced production of proinflammatory cytokines by inhibiting phosphorylation of MAPKs and STATs *in vitro* and *in vivo*.

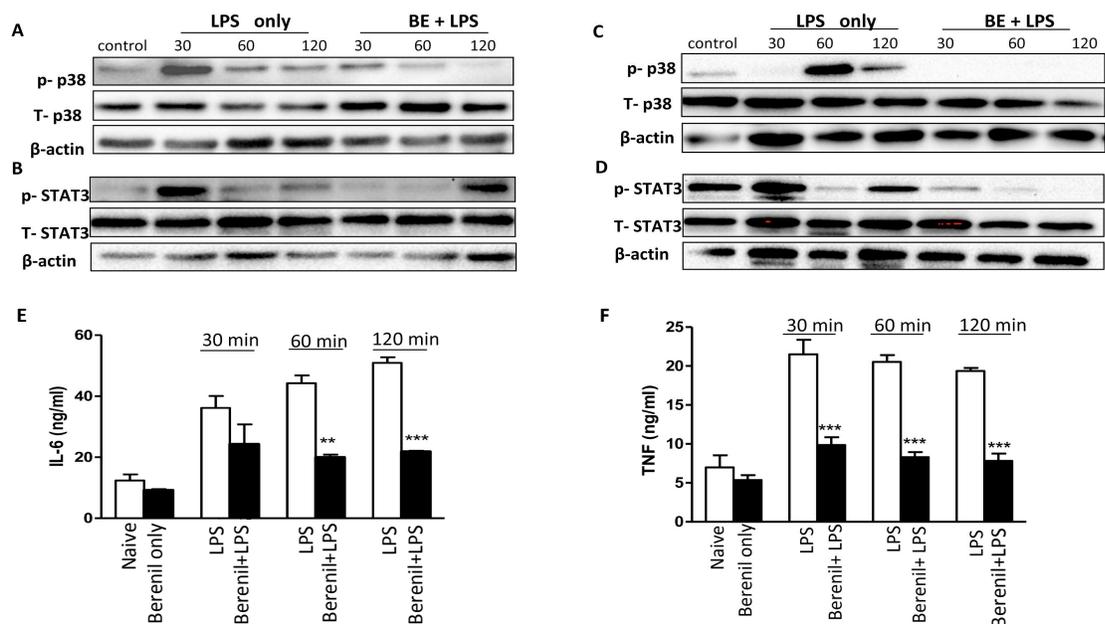


Figure 7.2.6. Berenil down-regulates MAPKs and STATs phosphorylation in peritoneal macrophages. Groups of mice were treated with or without Berenil (14 mg/kg) overnight and sacrificed to isolate peritoneal macrophages. The cells were further stimulated *in vitro* with LPS (1 μg/ml) for 30, 60 and 120 minutes, lysed and the lysates were assessed by western blot for phosphorylation of p38 (A) and STAT3 (B). The same blots were stripped and reprobbed with antibodies against total p38 and STAT3 and used as loading control. β-actin was also done as loading control. In another set of experiments, mice treated with or without Berenil (14 mg/kg) overnight were injected with LPS (10 mg/kg, i.p.) and at indicated times, sacrificed and lysates of peritoneal macrophages were assessed directly for phosphorylation of p38 (C) and STAT3 (D) by western blot. In addition, the levels of IL-6 (E) and TNF (F) in the peritoneal wash fluids were assayed by ELISA. The data presented are representative of two independent experiments with similar results (n = 3 mice per group). **, p < 0.01; ***, p < 0.001.

7.2.7 Berenil downregulates LPS-induced phosphorylation of NFκB p65 and IL-6 promoter activity

The NFκB/Rel family of transcription factors plays a crucial role in inflammation by their ability to induce transcription of proinflammatory cytokine genes. The most activated form of NFκB is a heterodimer consisting of p65 subunit and p50 subunit as they have the transactivation domains necessary for gene induction [316]. In order to evaluate whether Berenil suppresses proinflammatory cytokine production by altering NFκB activation and thus gene transcription, I performed western blot and ELISA to detect NFκB p65 phosphorylation. Pre-treatment of BMDMs with Berenil inhibited LPS-induced phosphorylation of NFκB p65 subunit as assessed by western blot (Figure 7.2.7A) and by ELISA (Figure 7.2.7B).

To further determine the mechanisms by which Berenil suppresses proinflammatory cytokines production, I investigated whether Berenil directly affects the promoter activity of IL-6 gene. ANA cells were transiently transfected with wild-type (WT) IL-6 Luciferase reporter constructs, pre-treated overnight with Berenil and stimulated with LPS. ANA cells transfected with IL-6 promoter construct showed a significant increase ($p < 0.01$) in luciferase activity in response to LPS (1 μg/ml) stimulation. In contrast, pre-treatment with Berenil dramatically downregulated LPS-induced promoter activity (Figure 7.2.7C). Collectively these data suggest that Berenil directly affects transcriptional mechanisms in macrophages leading to downregulation of proinflammatory cytokine production.

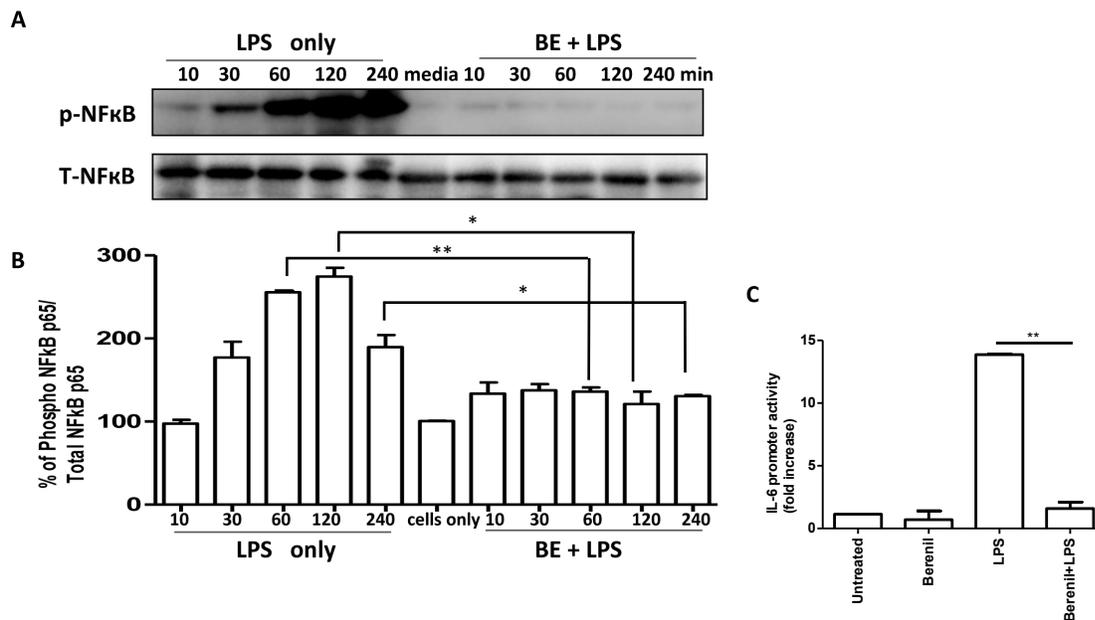


Figure 7.2.7. Berenil pretreatment downregulates LPS-induced phosphorylation of NFκB p65 subunit and IL-6 gene promoter activity in macrophages. BMDM were treated with Berenil (10 μg/ml) overnight and then stimulated with LPS (1 μg/ml). At indicated times, the cells were lysed and the lysates were assessed by western blot (A) or ELISA (B) for phosphorylation of NFκB p65 subunit. The same blot was stripped and reprobed with antibody against total NFκB and used as loading control (A). ANA-1 cells were transiently transfected with IL-6 promoter construct containing luciferase gene and then pretreated with Berenil (10 μg/ml) for 12 hr. Thereafter, the cells were stimulated with LPS (1 μg/ml) for another 12 hr and the luciferase reporter activity was measured as described in the materials and methods. Data are presented as fold increase over unstimulated controls are representative of 3 independent experiments with similar findings. *, $p < 0.05$; **, $p < 0.01$.

7.2.8 Pre-treatment with Berenil upregulates SOCS1 and SOCS3 expression in LPS stimulated macrophages

The suppressors of cytokine signaling (SOCS) are very important regulators of the immune system and have been shown to regulate activation and cytokine production by T cells, macrophages and dendritic cells. Forced expression of SOCS3 in a mouse arthritis model suppresses proinflammatory cytokine production and the induction or the development of the disease [317]. In addition, SOCS1 has been reported to block the initial steps involved in TLR signalling in macrophages [318]. Therefore, I investigated the possibility that Berenil might be suppressing proinflammatory cytokine production by upregulating SOCS expression. As shown in Figure 7.2.8 A-D, pre-treatment of BMDMs caused increased expression of SOCS1 and SOCS3 starting as early as 30 min and lasting up to 24 hr. These results suggest that the inhibitory effects of Berenil on MAPK and STAT phosphorylation and the suppression of proinflammatory cytokine production may be due to its effect on SOCS induction.

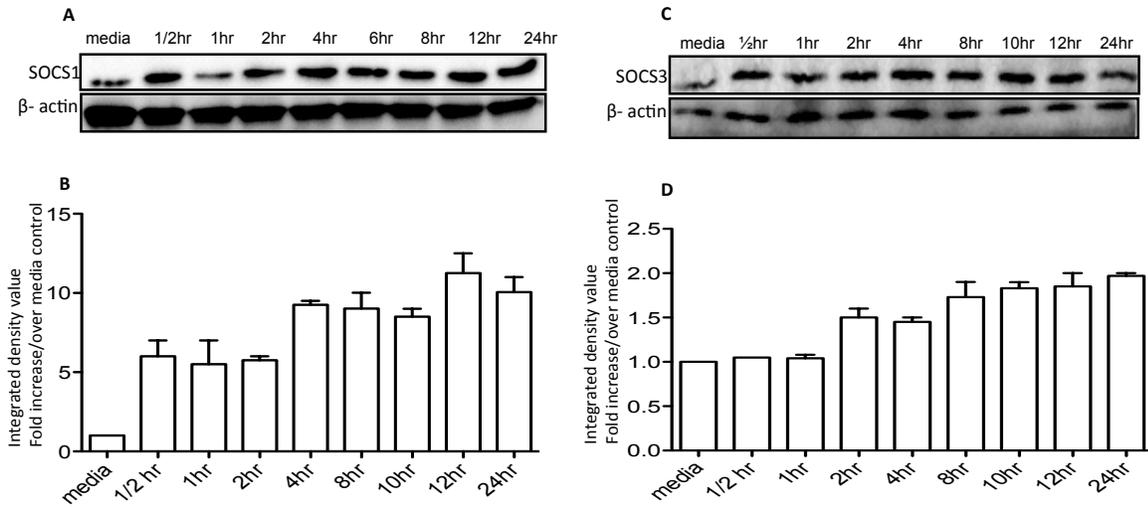


Figure 8. Berenil induces SOCS1 and SOCS3 expression in macrophages. BMDM were treated with Berenil (10 $\mu\text{g/ml}$) and at designated time points the cells were lysed and SOCS1 and SOCS3 expression were assessed by immunoblotting of total cell lysates using anti-SOCS1 and SOCS3 antibodies. The same blot was stripped and reprobed with antibody against β -actin as loading control. The data presented are representative of 2 independent experiments with similar findings.

CHAPTER 8- DISCUSSION

8.1 Diminazene aceturate (Berenil) modulates the host cellular and inflammatory responses to *Trypanosoma congolense* infection.

The primary objective of this study was to determine whether diminazene aceturate (Berenil) has, in addition to its trypanolytic property, an immunomodulatory effect following infection with *Trypanosoma congolense*. Acute death of *T. congolense*-infected susceptible (BALB/c) mice has been attributed to immune hyper-activation (particularly of macrophages and T cells), which leads to concomitant over production of pro-inflammatory cytokines, SIRS, immunopathology and death [10]. In addition, regulatory T cells have been implicated in enhanced susceptibility to experimental *T. congolense* infection [285]. In this study, I showed that Berenil treatment significantly lowers the serum levels of pro-inflammatory cytokines in both the highly susceptible and relatively resistant mice. Furthermore, I showed that Berenil treatment dampens immune cell activation as evidenced by significantly lower percentages of CD25 expressing lymphocytes in spleens of infected mice. Collectively, these observations suggest that survival of *T. congolense*-infected highly susceptible BALB/c mice following Berenil treatment may be related in part to the dampening of immune activation and pro-inflammatory cytokine production.

In addition to lower immune activation, I also found that the percentage of CD4⁺Foxp3⁺ (Treg) cells was significantly lower in infected mice after Berenil treatment (Figure. 4.2.1C and F). I speculate that the lower systemic inflammatory response in Berenil-treated mice may result in a lower necessity for Tregs leading to their impaired proliferation via unknown feedback mechanisms. The role of Tregs in experimental *T. congolense* infection is controversial. They have been shown to prevent effective control of parasitemia such that

when BALB/c mice were treated with low doses of anti-CD25 depleting antibody (PC61), they became resistant to *T. congolense* infection [285]. In contrast, another study found that IL-10-producing FoxP3⁺ Tregs are required for decreasing excessive macrophage activation [286]. I recently found that treatment of mice with PC61 prior to infection leads to an increased prepatent period and reduced peak parasitemia in both the highly susceptible and relatively resistant mice [319]. In fact, depletion of Tregs by a single treatment of anti-CD25 mAb prior to infection resulted in clearance of first wave of parasitemia and significantly increased the survival period in the highly susceptible BALB/c mice that normally do not control first wave of parasitemia [319]. Thus, it is conceivable that the enhanced resistance in mice following treatment with Berenil may in part be related to its effect in lowering Tregs in infected mice. Interestingly, Berenil treatment did not alter the frequency of Tregs in naïve mice, contrary to the observation in *T. congolense*-infected mice. This suggests that the reduction in Treg numbers observed in infected and treated mice might be related to infection-induced increase in numbers of these cells. However, Berenil treatment caused a significant reduction in serum levels of IL-6 and TNF in naïve animals (see Figure 4.2.2 I and J). Collectively, these observations suggest that the down-regulatory effect of Berenil on pro-inflammatory cytokine production is distinct and not related to its effect on Tregs.

I found that Berenil treatment also caused significant reduction in serum IL-10 levels in infected mice. Although IL-10 has a well-documented anti-inflammatory role in *T. congolense* infection, it has also been shown to mediate trypanosome-induced immunosuppression [77, 280, 320, 321]. Previous work has shown that following Berenil treatment of infected mice, immunosuppression is alleviated and mice were able to successfully respond to secondary bacterial infection [250]. It is possible that in addition to clearing parasites, Berenil contributes

to restoring immune responses by decreasing systemic overproduction of IL-10. Alternatively, it is possible that Berenil has a global suppressive effect on cytokine release by activated macrophages. In addition to IL-6, TNF- α and IL-12, macrophages produce copious amounts of IL-10 following their interaction with trypanosomal antigens [321]. In deed, in subsequent studies, I showed that that Berenil suppresses LPS- and CpG-induced pro-inflammatory cytokine responses by bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) *in vitro*; an effect that is mediated via suppression of MAP kinases and STATs signaling pathways (see chapter VII). Thus, if Berenil globally alters cytokine production in macrophages, this will also affect IL-10 levels as observed in this study.

Clearance of parasites from the blood of *T. congolense*-infected mice is primarily mediated by macrophages particularly liver (kupffer cells) and splenic macrophages [175]. The uptake of parasites coated with antibodies (including IgM and IgG) results in macrophage activation leading to the production of pro-inflammatory cytokines [10, 81]. In addition, activated macrophages also present trypanosomal antigens and stimulate T cells in an MHC class II-dependent manner to produce IFN- α , which further activates macrophages leading to more cytokine production. Thus, massive phagocytosis of trypanosomes (as seen during peak parasitemia) leads to hyper-activation of macrophages and increased production of monokines (IL-1, TNF- α , IL-6, IL-12, monocyte chemotactic protein-1 [MCP-1]) and the T-cell cytokine (IFN- γ). This systemic barrage of cytokine production leads to SIRS and proves fatal to the mouse. I found that in addition to immune hypoactivation, Berenil treatment also significantly lowers serum levels of pro-inflammatory cytokines and suppresses the production of pro-inflammatory cytokines by splenic CD11b⁺ and kupffer cells following LPS stimulation. This suggests that either the macrophage activation machinery or their ability to sense and respond

to external stimuli had been altered *in vivo*. Indeed, I found that in uninfected animals, Berenil lowers serum levels of pro-inflammatory cytokines and dampens LPS-induced septic shock following *in vivo* LPS injection. I hypothesize that the dampening of macrophage activation and production of pro-inflammatory cytokines (due probably to direct inhibition of intracellular signaling pathways (MAPK and STATs), reduces the impact of SIRS leading to improved prognostic outcome for the host.

IFN- γ is particularly important in *Trypanosome*-induced cytokine storm. Studies have shown that treatment of BALB/c mice with anti-IFN- γ antibodies prevents acute death in these mice [77]. On the other hand, IFN- γ is necessary for protection because the relatively resistant C57BL/6 mice treated with anti-IFN- γ antibodies become susceptible and die from fulminating parasitemia [71]. Furthermore, IFN- γ receptor deficient C57BL/6 mice succumb acutely to first wave of parasitemia [71]. Interestingly, treatment of the relatively resistant C57BL/6 mice with anti-IL-10R blocking antibody abrogates resistance, which is restored by combined treatment with anti-IFN- γ mAb [95]. These observations suggest that there is a fine balance between the effects of IL-10 and IFN- γ in *T. congolense* infected mice.

In conclusion, this section of the thesis elegantly showed that treatment of *T. congolense*-infected BALB/c mice with Berenil leads to dampening of T cell and macrophage hyper-activation, a lower percentage of FoxP3 regulatory T cells and lowering of systemic pro-inflammatory cytokine levels. These effects were directly associated with dampening of macrophage responses to microbial stimuli *in vitro* and *in vivo*. These findings suggest that in addition to its trypanolytic effects, Berenil also modulates the host immune response, and this may contribute to a more effective parasite control. It is unlikely that the decrease in parasite load resulting from the trypanolytic effect of Berenil is solely responsible for such a significant

reduction in pro-inflammatory cytokines and alteration in cellular immune responses given the fact that similar effects were observed in Berenil-treated uninfected mice and following LPS challenge *in vitro* and *in vivo*.

8.2 *Trypanosoma congolense*-induced pro-inflammatory cytokine production is by the activation of TLR signaling system through MAPK and STAT proteins.

Acute death of mice infected with African trypanosomes is usually associated with hyper-activation of immune cells (particularly macrophages and T cells), excessive production of pro-inflammatory cytokines (including IFN- γ , IL-6, IL-12 and TNF) and systemic inflammatory response like syndrome [74]. I previously showed that the production of pro-inflammatory cytokines by splenic and liver macrophages following *T. congolense* infection contributes to disease and treatment with Berenil dramatically suppressed the production of these cytokines and prevents death in the highly susceptible mice. The primary aim of the study performed in this section was to investigate the innate receptor and molecular mechanisms involved in *T. congolense* recognition and induction of pro-inflammatory cytokine production by macrophages. Using more controlled *in vitro* conditions, I showed directly that *T. congolense* induces proinflammatory cytokine production in both primary and immortalized macrophage. I further showed for the first time that TLR2 (but not TLR4) is the major innate receptor involved in *T. congolense*-induced cytokine production in macrophages and that adaptor protein MyD88 is critically involved in this process. Furthermore, I showed that MAPKs (p38, ERK and JNK) and STATS (STAT1 and STAT3) are involved in *T. congolense*-induced cytokine production. Blockade of MAPKs and STATs with their specific inhibitors abrogated proinflammatory cytokine production. Collectively, these observations

reveal the molecular and intracellular signaling events that lead to proinflammatory cytokine production in macrophages following their interaction with *T. congolense*.

Toll-like receptors (TLRs) are important group of innate immune receptors that help in the recognition of microbes by the innate immune cells (including macrophages) and initiation of innate immune response [212]. Recent reports show that TLRs play important role in initiating innate immune response against several parasitic infections including *T. brucei*, *T. cruzi*, *L. major* and *Toxoplasma gondii* [98, 229, 230, 233]. *T. cruzi*-derived glycosphosphatidylinositol (GPI) moieties that anchors the major surface glycoprotein have been shown to trigger NF κ B activation through TLR2. Thus, macrophages from TLR2 deficient mice failed to produce pro-inflammatory cytokines in response to GPI stimulation *in vitro* [295]. In addition, DNA from *T. cruzi* stimulates cytokine production in a TLR9-dependent manner and synergizes with parasite-derived GPI anchor for the cytokine induction in macrophages [233]. Another study showed that a subset of free GPI anchors from *T. cruzi* known as glycoinositolphospholipids, stimulates cytokine production in a TLR4-dependent and TLR2-independent manner [294]. In contrast, *P. falciparum* GPI induces TNF production in macrophages through TLR4, TLR2 and TLR1 [231, 232]. Hitherto, no study has investigated the role of TLRs in *T. congolense*-mediated cytokine production. The results of my studies show that the recognition of *T. congolense* leading to the activation of intracellular signaling events and production of proinflammatory cytokines in macrophages is mediated through TLR2 and is independent of TLR4.

The ligation of TLRs by their corresponding ligands leads to the recruitment of adaptor proteins and initiation of intracellular signaling events that ultimately results in proinflammatory cytokine gene expression. MyD88 is the most important adaptor molecule

that is centrally involved in the activation of downstream signaling events following ligation of several TLRs by their respective ligands. A recent report showed that deficiency of MyD88 leads to enhanced susceptibility to *T. brucei* infection, due in part to impaired IL-12 production by macrophages and a consequent impairment in Th1 response [98]. The activation of MyD88 results in activation and/or phosphorylation of key signaling pathways including STATs and MAPKs. I found that deficiency of MyD88 leads to impaired phosphorylation of MAPKs and STATs in macrophages following *T. congolense* stimulation and this was associated with dramatic suppression in proinflammatory cytokine production. This observation is consistent with a recent report showing that *T. brucei rhodesiense* sVSG initiates the expression of a number of pro-inflammatory genes such as TNF, IL-12, IL-6 and iNOS by activating the ERK, p38, JNK and NFκB pathways [67]. Similarly, the GPI anchor of *T. cruzi* trypomastogotes glycoprotein has been shown to trigger the phosphorylation of ERK and p38 in macrophages leading to the activation of NFκB induction of pro-inflammatory genes. Furthermore, *T. gondii* has also been shown to induce MAPK and STAT3 phosphorylation in macrophages. Thus, it appears that the activation of MAPKs and STATs is a common pathway that is shared by protozoan parasites for the initiation of inflammatory cytokine production in macrophages.

Although my *in vitro* and *in vivo* studies showed that TLR2 is critical for *T. congolense*-induced activation of MAPK and STAT proteins and the subsequent production of proinflammatory cytokines in macrophages, I observed that deficiency of TLR2 (as seen in TLR2^{-/-} mice) results in increased susceptibility to *T. congolense* infection. Thus, TLR2^{-/-} mice on the usually resistant background were unable to control their first wave of parasitemia and succumbed to the infection within 8-10 days post-infection. Paradoxically, this acute death was associated with increased serum levels of TNF and IFN-γ and a concomitant increase in the

numbers of CD4⁺ T cells producing these cytokines in the spleens and liver of infected TLR2^{-/-} mice. This finding suggests that TLR2-dependent immune activation may play a complex role in the overall immunity to *T. congolense* infection in mice. In macrophages (and perhaps other innate immune cells), TLR2-dependent recognition of *T. congolense* triggers cytokine responses that may be critical for initiating protective adaptive immunity that is required for effective parasitemia control. In contrast, TLR2 signaling may be critical for restraining excessive cytokine production by CD4⁺ T cells in infected mice, thereby preventing immunopathology and death.

Although the results from this study clearly show the importance of TLR2 in proinflammatory cytokine production and resistance to *T. congolense* infection, the parasite molecule that is recognized by this receptor remains to be defined. Recent studies have shown that the GPI of *T. cruzi* and *P. falciparum* can bind to TLR2 on macrophages leading to their production of proinflammatory cytokines [233, 322, 323]. Given that *T. congolense* variant surface glycoprotein is anchored to the cell membrane via a GPI anchor, it is conceivable that this molecule may be the critical ligand for TLR2 in our system. In line with this, I observed that *T. congolense*-induced MAPK and STAT phosphorylation and production of proinflammatory cytokines were unaffected in macrophages from TLR4 deficient mice, suggesting that TLR4 may not be important in this model. However, it is likely that the induction of proinflammatory cytokine production and activation of innate immune response following *T. congolense* infection may involve interactions between several TLRs that signal via MyD88. Further studies are required to clearly delineate the key parasite molecules involved in TLR2-MyD88 dependent resistance to *T. congolense* infection.

In conclusion, the results from these studies identify, for the first time, TLR2- and MyD88-dependent activation of MAPKs and STATs as key intracellular events that are involved in cytokine production and enhanced resistance *T. congolense* infection in mice. Deficiency of TLR2 leads to uncontrolled first parasitemia and acute death in an otherwise relatively resistance C57BL/6 mice. Understanding the receptors, adaptor proteins and the complex signaling pathways are of great interest and could eventually be used to develop novel strategies to stimulate protective immunity and prophylaxis against the infection.

8.3 Berenil pre-treatment down-regulates *T. congolense* (TC13)- induced IL-6 and IL-12 production in macrophage cell lines (ANA) and BMDM.

In the previous chapter, I identified important signaling molecules and pathways involved in *T. congolense*-induced proinflammatory cytokine production in macrophages. In addition, I had earlier shown that treatment of *T. congolense*-infected mice with Berenil leads to suppression of proinflammatory cytokine production, which is associated with survival from an otherwise lethal infection. Collectively, these studies present an important question: does Berenil inhibit *T. congolense*-induced proinflammatory cytokine production in macrophages and if it does, is this by downregulating the phosphorylation of MAPK and STAT proteins? The data presented in this chapter identify for the first time, the direct effect of Berenil pre-treatment in *T. congolense*-induced intracellular signaling pathways that lead to proinflammatory cytokine production in macrophages. Using macrophage cell lines and primary BMDMs, I found that Berenil pre-treatment significantly down-regulates *T. congolense*-induced IL-6 and IL-12 production in these cells. Then I tested the effect of Berenil pre-treatment on ERK, p38, STAT1 and STAT3 signaling pathways in response to *T. congolense* stimulation. Interestingly,

I found that the inhibitory effect of Berenil is not global and it did not alter TC-induced ERK phosphorylation whereas the p38, STAT1 and STAT3 phosphorylation was significantly down regulated. I further confirmed these observations by pre-treating mice with Berenil, inoculated them with TC lysate and assessed MAPK and STAT phosphorylation in peritoneal macrophages directly *ex vivo*. I found that as in BMDMs, Berenil pretreatment did not alter ERK phosphorylation, but downregulated the p38, STAT1 and STAT3 phosphorylation in response to TC in peritoneal macrophages.

NFκB is a crucial transcription factor for pro-inflammatory cytokine production and several parasites including *T. cruzi* have been shown to trigger NFκB activation through Toll-like receptor-2 [295]. GPI from *T. cruzi* have been shown to trigger the phosphorylation of ERK, p38 and also IκB, thereby leading to the activation of NFκB and subsequent activation of pro-inflammatory genes. *T. brucei* also have been shown to induce activation of ERK, p38, JNK and NFκB pathways resulting in the expression of a number of pro-inflammatory genes such as TNF, IL-12, IL-6 and iNOS [67]. In the previous chapter, I had also shown that *T. congolense* induces the activation of NFκB p65 and that Berenil pretreatment downregulates TC induced phosphorylation of NFκB. Interestingly, I found that Berenil also downregulated TC-induced IL-6 promoter activity, suggesting that the compound may directly alter proinflammatory cytokine gene transcription.

Although Berenil is the most commonly used therapeutic agent for treatment of animal trypanosomiasis for over 60 years, its molecular mechanisms of action is not completely known. The work presented here explains for the first time how Berenil blocks the signaling events responsible for NFκB-mediated macrophage activation and cytokine production in response to *T. congolense*. The present study shows that Berenil blocks p38, STAT1 and STAT3

phosphorylation in response to *T. congolense* in macrophages. Within the pharmaceutical industry, there is an increasing interest in targeting the MAPK and other intracellular pathways that regulate inflammatory responses. Thus, Berenil might be a viable and novel agent for use in the treatment of various inflammatory conditions. In addition, the therapeutic aspects of trypanosomiasis need alternative approaches as there is no vaccine and no new drugs are being developed and the current drugs have several shortcomings. A complete understanding of the mechanism of action of Berenil, particularly the mechanisms through which it blocks *T. congolense*-induced cytokine production could help increase the efficacy of the drug and reduce unwanted side effects.

8.4 Diminazene aceturate (Berenil) modulates LPS-induced proinflammatory cytokine production by inhibiting phosphorylation of MAPKs and STAT proteins.

In the previous chapters, I showed that Berenil treatment dramatically suppressed the production of proinflammatory cytokines (IL-6, IL-12p40 and TNF) by splenic and liver macrophages leading to a concomitant reduction in serum levels of these cytokines following *Trypanosoma congolense* infection or LPS challenge. Therefore, the primary aim of the studies conducted in this chapter was to investigate the molecular mechanisms through which Berenil alters proinflammatory cytokine production in macrophages. Using a more controlled *in vitro* conditions, I showed that pre-treatment with Berenil downregulated LPS-, CpG- and poly (I:C)-induced cytokine production in macrophages and dendritic cells. Because these pathogen-associated molecular patterns (PAMPs) are recognized by innate immune cells via TLRs, I hypothesized that Berenil downregulates TLR expression on macrophages. Surprisingly, I found that Berenil did not alter the expression of any of the TLRs tested (TLR2,

TLR4 and TLR9) on macrophages. Therefore, I considered the possibility that Berenil interferes with signaling molecules and transcription factors that mediate the expression of proinflammatory cytokines. Indeed, I showed that Berenil downregulates the phosphorylation of MAPKs (ERK, p38, JNK), STATs (STAT1 and STAT3) and NFκB p65 subunit, key signaling molecules and transcription factors involved in the production of proinflammatory cytokines by immune cells. In addition, I found that Berenil downregulated IL-6 promoter activity, indicating that the compound also directly affects transcription of IL-6 gene following LPS stimulation. Similar to the *in vitro* effects, we showed that Berenil also inhibits MAPK and STAT phosphorylation *in vivo*. Interestingly, I found that Berenil upregulated SOCS1 and SOCS3 expression, molecules that act as negative regulators of cytokine signaling by inhibiting MAPKs and STATs. This finding suggests that Berenil may be downregulating the activation of MAPK and STATs and hence proinflammatory cytokine production by enhancing the expression of SOCS proteins.

The hyper-activation of macrophages and a concomitant over production of proinflammatory cytokines has been proposed as the leading cause of acute death in *T. congolense*-infected highly susceptible BALB/c mice [10]. *Trypanosoma congolense*-infected BALB/c have significantly higher serum levels of IL-6, IL-12p40 and TNF than infected relatively resistant C57BL/6 mice [74]. The over production of proinflammatory cytokines in infected BALB/c mice leads to systemic inflammatory response syndrome-like phenomenon leading to death. In previous chapter, I found that treatment of *T. congolense*-infected mice with Berenil clears parasitemia and dramatically lowers serum levels of proinflammatory cytokines including IL-6, IL-12p40 and TNF. The dampening effect on proinflammatory cytokine production was mediated by the effect of Berenil on CD11b⁺ cells, including splenic

and liver macrophages. Furthermore, pre-treatment of mice with Berenil significantly ameliorated LPS-induced sepsis and proinflammatory cytokine production *in vivo*. In the present study, I found that Berenil downregulated LPS-, CpG-, and Poly (I:C)-induced cytokine production, strongly suggesting that the effect of the drug is global and is not pathogen-specific.

Macrophages are key innate immune cells that contribute to efficient destruction of intracellular pathogens via the production of free radicals and other potent cytotoxic molecules including nitric oxide. In addition, they also act to shape the overall immune response through the production of inflammatory and immunomodulatory cytokines. Once pathogens are recognized by macrophages, they are taken up by phagocytosis, which initiates a cascade of intracellular events that may lead to the production of proinflammatory cytokines. Various pattern recognition receptors, (including TLRs), are constitutively expressed by macrophages and play important roles in the recognition of specific microbial components [324]. In response to various stimuli, the TLRs are activated and this initiates the activation of powerful intracellular signaling pathways through their cytoplasmic TIR domains [325, 326]. Once the TLR signaling is initiated, several adaptor protein families are recruited in a finely coordinated manner, which in turn activates the downstream protein kinases ultimately leading to the activation of key transcription factors such as NF κ B or AP-1 [327]. These transcription factors then enter the nucleus and bind to their specific binding sites on the gene promoters leading to transcription of proinflammatory genes. I found that despite suppressing LPS-, CpG- and poly (I:C)-induced production of proinflammatory cytokines, Berenil did not alter the expression TLR4, TLR9 and TLR2 on macrophages. In addition, it did not alter MD2 and CD14 expression on macrophages. MD2 is intimately associated at the cell surface with TLR4 [328]

and plays a critical role in recognition and regulation of cell activation by LPS [329]. Similarly, CD14 is a key component of LPS recognition because it binds and transfers LPS to LPS binding protein (LBP), an important step that is crucial for LPS interaction and activation of TLR signaling events. Thus, our results show that the downregulation of proinflammatory cytokine production by Berenil is not due to its alteration of TLR expression on macrophages.

In response to inflammatory stimuli or following ligation of TLRs by their specific agonists, a series of intracellular signaling cascades are initiated that ultimately lead to activation of macrophages and their release of proinflammatory cytokines. Prominent among these intracellular signaling events include the phosphorylation of mitogen activated protein kinases (MAPK) and signal transducer and activation of transcription (STAT) pathways leading to the activation of NF κ B [330], a crucial transcription factor for proinflammatory cytokines production. Indeed, LPS-induced production of inflammatory cytokines by macrophages involves activation of MAPKs [331, 332]. In addition to activating the MAPK pathway, binding of LPS to TLR4 also activates the JAK-STAT signal transduction pathways, particularly STAT1 [333-335]. Several studies indicate that the anti-inflammatory effect of various naturally occurring as well as synthetic compounds are due to their effect on ERK, JNK, p38 and STAT phosphorylation. For example, silibinin treatment inhibits iNOS expression in tumor cells and TNF-induced production of proinflammatory cytokines in lung epithelial carcinoma cells by inhibiting phosphorylation of STAT1, STAT3 and ERK1/2 leading to a concomitant suppression of NF- κ B and AP-1 activation [336]. Another study indicates that curcumin suppresses the JAK-STAT pathway and thus attenuates inflammatory responses of brain microglial cells [337]. Furthermore, it has been reported that estradiol downregulates LPS-induced cytokine production in macrophages and this effect was mediated

via alteration of intracellular signaling pathways that results in impaired NFκB binding activity [338]. In line with these, our studies show that Berenil significantly impairs the phosphorylation of ERK, p38, JNK, STAT1, STAT3 and NFκB suggesting that it inhibits MAPKs and JAK-STAT signaling pathways. Such a wide range of inhibitory targets could explain the global effects of this compound in suppressing proinflammatory cytokines, including those associated with CpG and Poly (I:C) stimulation. Interestingly, I found that when macrophages were pre-treated with Berenil overnight and stimulated with LPS (1μg/ml) for only 1-2 hr, there was a downregulation of STAT1 and STAT3 phosphorylation without a concomitant downregulation of proinflammatory cytokine production. The downregulation of proinflammatory cytokine production was observed only after prolonged (> 12 hr) LPS stimulation, strongly suggesting that the reduced phosphorylation of STAT1 and STA3 is not likely due to reduction in cytokine production, but more likely related to the direct effect of Berenil on the cells. In contrast, I found that Berenil pre-treatment upregulated STAT5 phosphorylation in macrophages. Previous studies have shown that GM-CSF and IL-15 upregulate STAT5 phosphorylation in several cell types [339-341]. It is conceivable that the increased STAT5 phosphorylation following Berenil pre-treatment may be indirect, due in part to increased production of IL-15 and GM-CSF by macrophages.

How does Berenil inhibit LPS-induced MAPKs and STATs phosphorylation leading to suppression of proinflammatory cytokine production in macrophages? TLR signaling must be tightly regulated to maintain a balance between activation and inhibition of the immune system in order to avoid inappropriate inflammatory responses. Following the interaction of TLRs with their respective ligands, the ensuing intracellular signalling events are tightly controlled

by intracellular regulators, including suppressor of cytokine signalling (SOCS) proteins, TRIAD3A and A20, TOLLIP, etc. [342]. In particular, SOCS proteins have been shown to inhibit many intracellular signaling pathways including those mediated by MAPKs and STATs [343]. SOCS proteins inhibit signal transduction by acting in a negative feedback loop to dampen excessive activation [344]. Indeed, it has been shown that SOCS proteins inhibit LPS-induced IL-6 production by regulating the JAK-STAT pathway [345]. Another study showed that SOCS3 inhibits TNF-induced MAPK phosphorylation in rat islets [346]. I found that Berenil pre-treatment upregulates SOCS1 and SOCS3 expression in macrophages. I speculate that this upregulation of SOCS proteins by Berenil has a negative effect on STAT and MAPK signaling pathways, partially contributing to the downregulation of proinflammatory cytokine production in macrophages. In addition to upregulating SOCS1 and SOCS3, I also found that Berenil significantly inhibits LPS-induced NF κ B and IL-6 gene promoter activity suggesting that in addition to its effects on MAPKs and STATs, Berenil also directly affects transcriptional activities in macrophages. It is also conceivable that Berenil might, in addition to upregulating SOCS, affect the activities of other negative regulators in macrophages.

In summary, the collective results presented in this chapter demonstrate that Berenil modulates proinflammatory immune response by inhibiting key signaling pathways and transcription factors associated with inflammatory cytokine production. Thus, my studies point to a novel effect of Berenil. The suppression of proinflammatory cytokines production in macrophages by Berenil *in vitro* and *in vivo* suggests that this compound might have anti-inflammatory properties and could therefore be used for the treatment and control of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease,

atherosclerosis, sepsis etc. Unveiling the molecular mechanisms of action of Berenil could result in ways to increase the efficacy of the drug and prevent its unwanted side effects.

8.5. Missed opportunities and limitations

In Chapter IV, I identified the immuno modulatory properties of Berenil. Treatment of infected mice with Berenil results in dampening of T cell and macrophage hyper-activation and lowers systemic pro-inflammatory cytokine levels. These are associated with the therapeutic effects of Berenil. It would have been interesting to see whether there is any prophylactic effect for this drug and whether other the immune cells are affected in a similar way. Pre-treating mice with Berenil before infection with *T. congolense* and testing the activation of T cells and macrophages and serum cytokine levels would provide more information about the effect of pre-treatment of the drug and would have provided a stronger data.

In an attempt to identify the innate receptor and signaling molecules associated with *T. congolense* infection, I used BMDMs for *in vitro* studies and peritoneal macrophages for *in vivo* studies. Although it has been clearly documented that macrophages are vital for both clearance of *T. congolense* and in mediating immunopathology in infected mice, liver macrophages are primarily associated with clearance of trypanosomes in infected mice. The data of the thesis would have been strengthened if I had used liver macrophages in our *ex vivo* experiments to investigate the signaling pathways associated with *T. congolense* induced cytokine production. Furthermore, although I clearly demonstrated that TLR2 is involved in *T. congolense* recognition and immunity following infection, the parasite molecule that is involved in the recognition was not identified. Identification of the molecule would be desirable and could provide targets for chemotherapy or vaccine design.

In chapter VII, we used both BMDM and BMDC to investigate the effect of Berenil treatment on LPS-induced proinflammatory cytokine production. As with bone marrow BMDM, Berenil treatment also downregulated LPS-induced cytokine production in BMDC. Thereafter, I did not further investigate the impact of Berenil on DCs in details as was done with macrophages. It would have been interesting to determine in details the effect of Berenil on DCs following LPS stimulation. Specifically, it would be necessary to determine whether the effect on cytokine production is mediated via the effects on intracellular signaling molecules and pathways. More importantly, I showed that Berenil treatment upregulated the expression of SOCS1 and SOCS3, key regulatory proteins that have been shown to suppress MAPK and STAT-induced proinflammatory cytokine production in macrophages [345] [346]. The data would have been strengthened if I knocked down SOCS1 and SOCS3 by siRNA to definitively determine whether phosphorylation of MAPK and STAT proteins and subsequent cytokine production in LPS-treated cells following Berenil treatment are rescued. It would have been also interesting to look for the expression of other intracellular regulators of cytokine production in macrophages like TOLLIP, TRIAD3A and whether their expression is upregulated upon Berenil treatment.

8.6 Therapeutic implications and treatment strategies

Inflammation is a host immune response that is triggered by infection or tissue injury. Inflammation is beneficial if it is highly regulated. However, prolonged and/or dysregulated inflammation leads to chronic inflammatory state and contributes to the pathogenesis of several chronic diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis etc. [301]. Macrophages play very important role in the initiation, maintenance and resolution of inflammation via antigen presentation, expression of co-stimulatory molecules and production of proinflammatory cytokines such as IL-6, IL-12, TNF etc [304]. In response to inflammation, cellular receptors are engaged by binding to these cytokines triggering intracellular signaling pathways like MAPKs and STATs that lead to activation of NF κ B and production of proinflammatory cytokines. Therefore, regulation of inflammatory signaling pathways is very important for the control of many inflammatory diseases. The involvement of signaling molecules in inflammation (such as MAPKs and STATs) makes them potential target for anti-inflammatory therapy. In this regards, several inhibitors of MAPKs have been developed with the sole aim of treating inflammatory conditions. For example, specific inhibitors of p38 (such as SB203580 and SB220025) have been shown to be effective in the prevention and treatment of murine model of collagen-induced arthritis [347]. Another selective p38 inhibitor, SB 242235, inhibits TNF- α and has protective effects against antigen-induced arthritis [348]. There are also reports showing that p38 inhibitors may be beneficial in the treatment of chronic inflammatory bowel disease, brain inflammation and animal models of stroke [349, 350]. CNI-1493, another potential anti-inflammatory drug, inhibits phosphorylation of both p38 and JNK. It can suppress macrophage activation and production of several proinflammatory cytokines and is shown to have protective effects in animal models

of endotoxic shock and rheumatoid arthritis [351, 352].

There is increasing interest within the pharmaceutical industry in targeting the MAPK and other intracellular signaling pathways that regulate inflammatory responses for therapeutic purposes. For example, newer anti-inflammatory therapies for rheumatoid arthritis include small molecule compounds such as inhibitors of MAPKs, cytokines and cytokine receptors. In particular, several p38 inhibitors have been developed and have been shown to be efficacious in preclinical management of some autoimmune disease models. Compounds such as RWJ 67657, VX-745 and BMS-582949 (all inhibitors of p38 MAPK) have shown significant improvement in patients with rheumatoid arthritis. Other p38 inhibitors such as SB235699 (HEP689) are in different stages of clinical trials as a topical agent for the treatment of psoriasis [353]. Similar to p38, small molecule inhibitors targeting the ERK pathway (such as PD184352, PD0325901 and ARRY-142886) have also been developed and some are at various stages of clinical trials [354]. Some compounds that possess anti-STAT inhibitory activities such as OPB-31121 (which is a STAT3 inhibitor), have also shown some promising antitumor activities and is currently in phase II clinical trial [355]

Berenil has been widely used for treatment of African trypanosomiasis and babesiosis (Table I). Given the global inhibitory effects of Berenil on MAPK and STAT activation and proinflammatory cytokine production *in vitro* and *in vivo*, I believe that the compound may be an attractive agent for treatment of diseases caused by excessive inflammatory cytokine production such as sepsis and autoimmune inflammatory conditions including rheumatoid arthritis, inflammatory bowel disease and uveitis (Table I).

Table 1.**Current and potential new therapeutic and clinical applications of diminazene aceturate (Berenil).**

Current application: anti-infective agent	References
1. Animal trypanosomiasis (<i>T. congolense</i> , <i>Trypanosoma vivax</i> , <i>Trypanosoma brucei brucei</i> , <i>Trypanosoma evansi</i> , <i>Trypanosoma equiperdum</i>)	[243-245, 247]
2. Babesiosis	[244]
Potential new application: anti-inflammatory agent	
1. Sepsis	See chapter 4&7
2. Uveitis	[356]
3. Glaucoma	[261]
4. Pulmonary hypertension	[259]
5. Ischemic stroke	[260]
6. Autoimmune and chronic inflammatory conditions (rheumatoid arthritis, inflammatory bowel disease, etc.)	^a chapter 7

^a. Indicative.

The global inhibitory effects of Berenil on cytokine production via selective inhibition of MAPK and STAT phosphorylation suggest that there is a strong potential for this drug to be used as kinase inhibitor to interfere with inflammation. Further studies are required to determine whether Berenil has any effect on other signaling pathways involved in inflammation, such as phospholipases and arachidonic acid cascades. In addition, the finding that Berenil can enhance ACE II activity suggests it may have possible therapeutic benefits for other diseases that involve the alteration in several physiological conditions such as pulmonary hypertension, ischemic shock and glaucoma. Thus, I propose that this old drug may have potentially novel benefits in the management of inflammatory disease disorders and altered physiological conditions.

Although it is known that *Trypanosoma congolense* infection in mice is associated with increased production of pro-inflammatory cytokines by macrophages and monocytes, the intracellular signaling pathways leading to the production of these cytokines has remained unexplored. In this thesis I investigated the innate receptors and intracellular signaling pathways that are involved in *T. congolense*-induced pro-inflammatory cytokine production in macrophages. These studies identified TLR2 and MyD88 dependent activation of MAPKs and STATs as key intracellular events responsible for cytokine production and enhanced resistance to *T. congolense* infection in mice. Targeting these pathways may be beneficial for treating animals with African trypanosomiasis, particularly those caused by *T. congolense*. Understanding the receptors, adaptor proteins and signaling pathways could be used to develop novel strategies against the infection.

Collectively, the in-depth studies I conducted in this thesis (and those of others) demonstrate that in addition to its trypanolytic effects, Berenil modulates the host immune response by inhibiting key signaling molecules and transcription factors associated with proinflammatory cytokine production. I believe that these data are strong enough to identify and perhaps advocate new uses for this old drug. Several drugs are used today in the clinics for the treatment of conditions that were not the primary intended use of the compound. The suppression of proinflammatory cytokines production in macrophages both *in vivo* and *in vitro* suggests that this compound have strong and potent anti-inflammatory properties. In addition, the demonstrated ability of Berenil to enhance the ACE II activity suggest that it could provide therapeutic benefits for treatment and control of chronic inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, sepsis and several other alterations of physiological conditions.

8.7 Immune modulation by Berenil and the potential mechanisms of action:

Although several reports suggest that Berenil might have immunomodulatory properties, no study has directly tested and/or demonstrated this empirically. To directly determine whether Berenil has any modulatory effect on host immune system, I examined several immune parameters in mice infected with *T. congolense* and treated with Berenil. I found that Berenil treatment significantly lowers serum levels of pro-inflammatory cytokines including IL-6, IL-12 and TNF in both the highly susceptible BALB/c mice and the relatively resistant C57BL/6 mice. These effects were mediated by Berenil's action on splenic and hepatic (kupffer cells) macrophages. Since acute death observed in *T. congolense*-infected BALB/c mice is due in part to the excessive production of pro-inflammatory cytokines leading to cytokine storm and systemic inflammatory response syndrome (SIRS) [10], I hypothesized that the beneficial effects of Berenil could be partly related to its ability to suppress proinflammatory cytokine production leading to dampening of SIRS. In line with this, I found that pretreatment of mice with Berenil ameliorates LPS-induced acute inflammation and death. This is an important finding because it shows for the first time that Berenil pre-treatment could dampen systemic inflammatory response by altering responsiveness of immune cells to microbial stimuli. Collectively these findings suggest that in addition to its trypanolytic effect, Berenil also modulates the host immune response by regulating inflammatory response, which may contribute to a more effective disease control.

Next, I used both *in vitro* and *in vivo* models of microbial molecule-induced proinflammatory cytokine induction in macrophages to fully elucidate the molecular mechanisms through which Berenil alters proinflammatory cytokine production. I showed that

pre-treatment of bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) with Berenil downregulated LPS, CpG and Poly I:C-induced proinflammatory cytokine production, suggesting that it may be affecting common intracellular pathways through which these molecules stimulate proinflammatory cytokine production. In support of this, I found that Berenil did not alter the expression of different TLRs, (including TLR2, TLR4 and TLR9) on macrophages and DCs when assessed by flow cytometry. Instead, it dramatically downregulated the phosphorylation of MAPKs (ERK, p38 and JNK), STATs (STAT1 and STAT3) and NFκB p65 subunit, which are key signaling molecules and transcription factors involved in the production of proinflammatory cytokines [299, 315]. I also demonstrated the downregulatory effect of Berenil on MAPK and STAT phosphorylation *in vivo* following intraperitoneal injection of LPS by showing that these mice displayed lower disease score and suppressed proinflammatory cytokine production. Interestingly, Berenil upregulated SOCS1 and SOCS3 expression, proteins that act as negative regulators of cytokine signaling and have been shown to inhibit intracellular signaling pathways mediated by MAPKs and STATs [343]. Hence I proposed that the induction of SOCS proteins by Berenil may be the main pathway involved in its suppressive effector on gene transcription and inflammatory cytokines production in macrophages. The following cartoon shows a schematic representation of the potential pathways targeted by Berenil to suppress inflammatory cytokine production in macrophage (Figure 8.6.1)

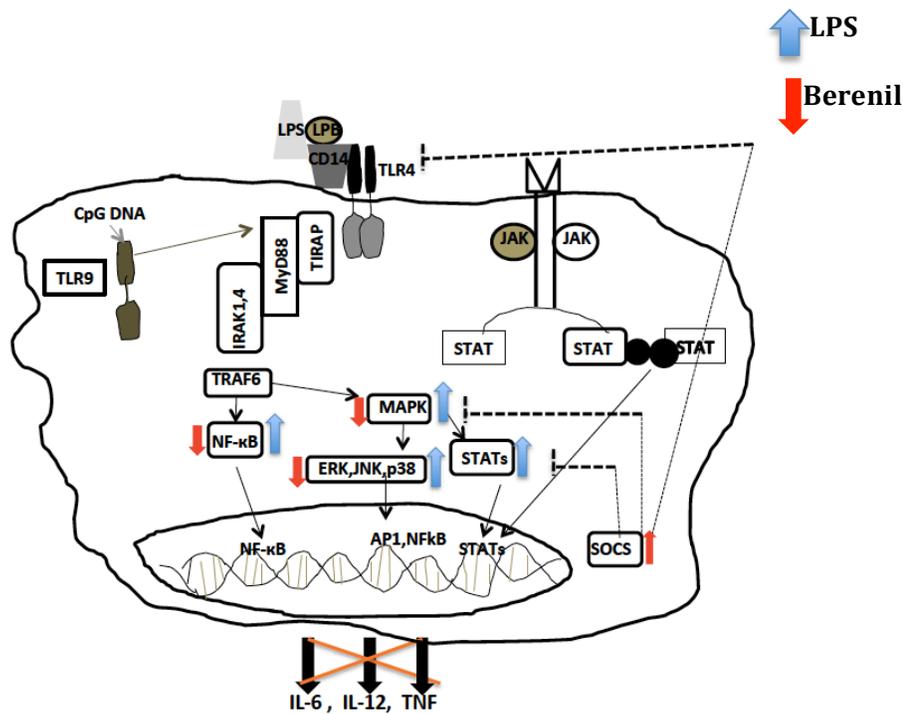


Figure 8.6.1 Proposed mechanisms through which Berenil downregulates LPS-induced cytokine production in macrophages. Binding of LPS to its receptor complex made up of TLR4, CD14 and LPS-binding protein on macrophages initiates intracellular signaling events that lead to the activation of several adaptor proteins including TIRAP, MyD88 and TRAF6. This in turn leads to the activation of MAP kinases (p38, ERK, JNK), STATs (STAT1 and STAT3) and phosphorylation and subsequent nuclear translocation of NFκB resulting in transcription of key proinflammatory cytokine genes (IL-6, IL-12 and TNF). Berenil treatment downregulates the phosphorylation of MAPKs and STATs, which in turn inhibits the activation and nuclear translocation of NFκB. Berenil also upregulates the phosphorylation of SOCS proteins (SOCS1 and SOCS3), which directly or indirectly regulate STAT, MAPK and TLR signaling pathways resulting in the downregulation of proinflammatory cytokine production

I found that following interaction with *T. congolense* there is increased cytokine production in macrophages, which is dependent on phosphorylation of MAPKs and STAT proteins. I also showed that *T. congolense* induced cytokine production in macrophages is mediated by Toll-like receptor 2. Berenil treatment downregulates *T. congolense* induced cytokine production in macrophages by affecting the phosphorylation of MAPKs and STAT proteins. The following cartoon shows a schematic representation of important innate immune receptors, adaptor proteins and signaling pathways involved in *T. congolense* induced cytokine production and how Berenil suppress it (Figure 8.6.2)

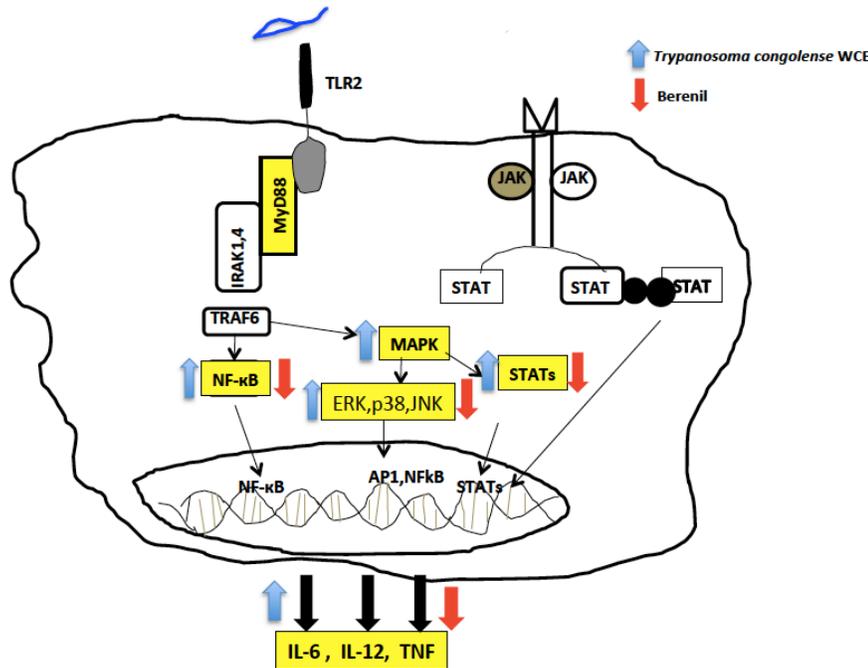


Figure 8.6.2 Signaling events involved in *T. congolense* induced proinflammatory cytokine production and how Berenil downregulates this: Binding of *T. congolense* to TLR2 in macrophages initiates intracellular signaling events that lead to the activation of the adaptor protein including MyD88. This in turn leads to the activation of MAP kinases (p38, ERK, JNK), STATs (STAT1 and STAT3) and phosphorylation and subsequent nuclear translocation of NFκB resulting in transcription of key proinflammatory cytokine genes (IL-6, IL-12 and TNF). Berenil treatment downregulates the phosphorylation of MAPKs and STATs, which in turn inhibits the activation and nuclear translocation of NFκB.

8.8 Major findings and conclusions

8.8.1 Effect of Berenil treatment on host cellular and inflammatory response to *T. congolense* infection.

In this part of the thesis I investigated whether Berenil has, in addition to its trypanolytic effect, a modulatory effect on the host immune response to *Trypanosoma congolense*. Data from this study show that Berenil treatment significantly reduced the percentages of regulatory (CD4⁺Foxp3⁺) T cells and caused a striking reduction in serum levels of disease exacerbating pro-inflammatory cytokines including IL-6, IL-12, TNF and IFN- γ . Furthermore, Berenil treatment significantly suppressed spontaneous and LPS-induced production of inflammatory cytokines by splenic and liver macrophages. The most intriguing data from this section of the thesis is finding that that Berenil treatment significantly ameliorated LPS-induced septic shock and the associated cytokine storm. Collectively, findings provide first direct and empirical evidence that in addition to its direct trypanolytic effect, Berenil also modulates the host immune response to the parasite in a manner that dampen excessive immune activation and production of pathology-promoting pro-inflammatory cytokines. Collectively, the findings suggest that Berenil may also be beneficial for treatment of disease conditions caused by excessive production of inflammatory cytokines. These finds were published in a peer review journal. **Kuriakose S**, Muleme H, Onyilagha C, Singh R, Jia P, Uzonna JE. Diminazene aceturate (Berenil) modulates the host cellular and inflammatory responses to *Trypanosoma congolense* infection. PLoS One. 2012;7. (11):e48696. doi: 10.1371/journal.pone.0048696. Epub 2012 Nov 7.

8.8.2 The innate immune receptors and signaling pathways involved in *T. congolense*-induced pro-inflammatory cytokine production.

In this part of the thesis, I explored the role of innate receptors and intracellular signaling pathways that are involved in *T. congolense*-induced pro-inflammatory cytokine production in macrophages. I showed that the production of IL-6, IL-12 and TNF- α in macrophages *in vitro* and *in vivo* following interaction with *T. congolense* is dependent on phosphorylation of mitogen-activated protein kinase (MAPK) including ERK, p38, JNK and signal transducer and activation of transcription (STAT) proteins. Specific inhibition of MAPKs and STATs signaling significantly inhibited *T. congolense*-induced production of proinflammatory cytokines in macrophages. I further showed that *T. congolense*-induced pro-inflammatory cytokine production in macrophages is triggered by the ligation of Toll-like receptor 2 (TLR2) and is dependent on signals mediated via the adaptor molecule, MyD88. Deficiency of TLR2 leads to impaired cytokine production by macrophages *in vitro* and acute death in otherwise relatively resistant mice. This is the first study that determined the critical innate receptor and the downstream intracellular signaling pathways involved in *T. congolense* recognition and induction of proinflammatory cytokine production following infection. The results provide mechanistic insights into *T. congolense*-induced activation of the innate immune system that leads to the production of pro-inflammatory cytokines and resistance to the infection. Understanding the innate immune receptors and signaling molecules involved in *T. congolense* infection may help to identify novel targets for immunomodulation aimed at regulating the disease. The findings from this section of the thesis is ready for submission. **Shiby Kuriakose**, Chukwunonso Onyilagha, and Jude E. Uzonna. *Trypanosoma congolense* induced pro-inflammatory cytokine production is by the activation of TLR signaling system through

MAPK and STAT proteins.

8.8.3 Effect of Berenil treatment in *T. congolense*-induced pro-inflammatory cytokine production

In this part of the thesis, I investigated the molecular mechanisms through which Berenil alters the *T. congolense*-induced cytokine production in macrophages. Data from this study showed that pre-treatment of macrophages with Berenil significantly down-regulated the phosphorylation of mitogen activated protein kinase (p38), signal transducer and activator of transcription (STAT) proteins including STAT1 and STAT3 and NFκB activity both *in vitro* and *in vivo*.

Results from this section indicate that Berenil down-regulates *T. congolense* induced cytokine production in macrophages by inhibiting the key signaling molecules and pathways associated with cytokine production. The findings from this study have been put into a manuscript that is ready for submission. **Shiby Kuriakose**, Chukwunonso Onyilagha, and Jude E. Uzonna. Diminazene aceturate (Berenil) downregulates *Trypanosoma congolense* induced pro-inflammatory cytokine production by altering the phosphorylation of MAPK and STAT proteins.

8.8.4 Modulation of LPS induced pro-inflammatory cytokine production by Berenil

In the last aspect of the thesis, I wanted to answer an important question: Is the downregulatory effects of Berenil on proinflammatory cytokine production specific to *T. congolense* or global (affecting different microbial products known to induce proinflammatory cytokine production)? Therefore, I used different TLR agonists including LPS, CpG and Poly I:C in

these studies. I showed that pretreatment of macrophages with Berenil dramatically suppressed IL-6, IL-12 and TNF production following LPS, CpG and Poly I:C stimulation without altering the expression of Toll-like receptors (TLRs). Instead, it significantly downregulated phosphorylation of mitogen-activated protein kinases (p38, ERK and JNK), signal transducer and activator of transcription (STAT) proteins (STAT1 and STAT3) and NFκB p65 activity both *in vitro* and *in vivo*. Interestingly, Berenil treatment upregulated the phosphorylation of STAT5 and the expression of SOCS1 and SOCS3, which are negative regulators of innate immune responses including MAPKs and STATs. Altogether, these results show that Berenil downregulates macrophage proinflammatory cytokine production in response to ligation of different TLRs suggesting that the effect is global. This implies that the drug may be used to treat conditions caused by excessive production of inflammatory cytokines. The findings from this section was published in *Innate Immunity*. **Kuriakose S**, Muleme H, Onyilagha C, Okeke E, Uzonna JE.

Diminazene aceturate (Berenil) modulates LPS induced pro-inflammatory cytokine production by inhibiting phosphorylation of MAPKs and STAT proteins. *Innate Immun.* 2014 Oct;20(7):760-73. doi: 10.1177/1753425913507488. Epub 2013 Oct 31.

CHAPTER 9. Future Directions

9.1 1 Effect of Berenil treatment on host cellular and inflammatory response to *T. congolense* infection.

Overall, the work presented here represents a significant contribution to the immunobiology of African trypanosomiasis and enhanced our understanding of the impact of Berenil on the immune system. In particular, I show that Berenil has a global inhibitory effect on proinflammatory cytokine production and MAPK and STAT phosphorylation, suggesting that it may be an attractive agent for treatment of diseases caused by excessive proinflammatory cytokine production such as sepsis, rheumatoid arthritis, inflammatory bowel disease etc. Therefore, it would be really interesting to see whether Berenil has any therapeutic and/or prophylactic effects in these conditions, particularly in experimental models of rheumatoid arthritis and inflammatory bowel disease.

Although the results provide information about the regulation of host immune responses in treated animals, the information may not have application to human infections due to Berenil-related side effects in humans. However despite its toxicity, Berenil has been (and may indeed still being) used in many poor and disease endemic countries because of its relatively low cost compared to other drugs. Therefore, a detailed understanding of the mechanism associated with its toxicity is important. It would be really interesting to see how Berenil is taken up by macrophages and what other proteins interact with or affected by such uptake. Tagging of Berenil or using radiolabelled Berenil might help to understand the how the compound is taken up and how it affects cytokine production.

Pre-treatment of mice with Berenil ameliorated LPS-induced toxicity, which was associated with reduction in disease score and significant reduction in serum cytokine levels. This is a

very exciting finding and in this experiment, I pretreated mice with Berenil overnight and then challenged with high dose of LPS (5 mg/kg). This prophylactic approach rarely happens in real life situation, as in most cases of sepsis, treatment is only initiated after onset of the disease. It would be really interesting to investigate whether Berenil can reverse an already established sepsis by injecting mice with high dose of LPS and treating them with Berenil several hours before the onset of clinical disease. In addition, it would be interesting to determine the cell types involved in this process by looking at the peritoneal wash fluid or spleen.

In this thesis I determined that *T. congolense*-induced proinflammatory cytokine production in macrophages is triggered by the ligation of TLR2 and the resulting downstream signals involves the adaptor protein MyD88. However I did not identify the specific parasite molecule that is recognized by this receptor. It would be really interesting to test the proinflammatory cytokine production by purified VSG, parasite DNA and synthesized GPI molecules in order to determine which parasite molecule is recognized by TLR2. I also found that infection of TLR2^{-/-} mice with *T. congolense* leads to uncontrolled first parasitemia and acute death in an otherwise relatively resistance WT (C57BL/6) mice. Paradoxically, deficiency of TLR2 was associated with increased IFN- γ and TNF production by CD4⁺ T cells in spleens and livers of infected animals. I used macrophages throughout the *in vitro* studies to investigate *T. congolense*-induced cytokine production and signaling pathways associated with it. Therefore, it would be really interesting to monitor the changes in macrophages and cytokine production *in vivo* or at least directly *ex vivo* in these organs following *T. congolense* infection of TLR2 deficient mice.

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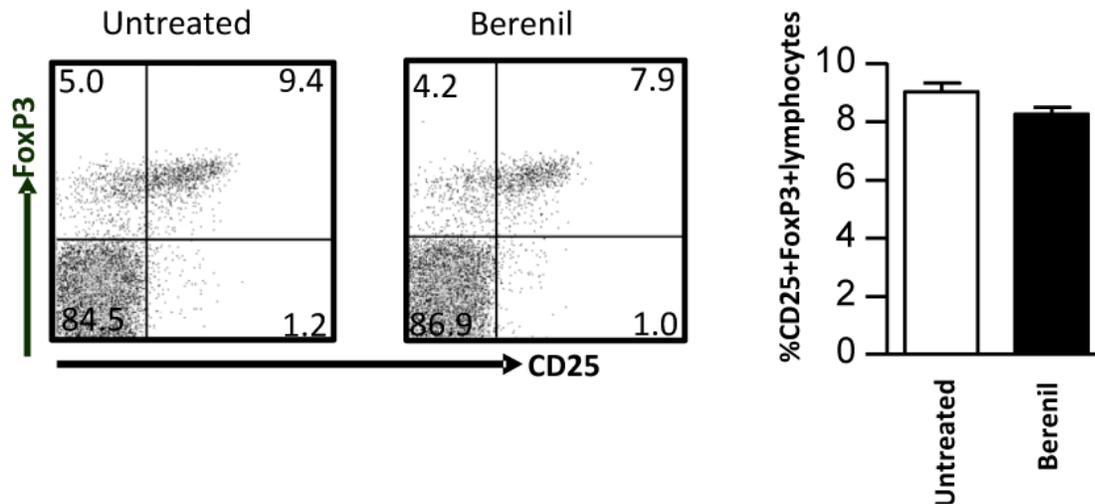
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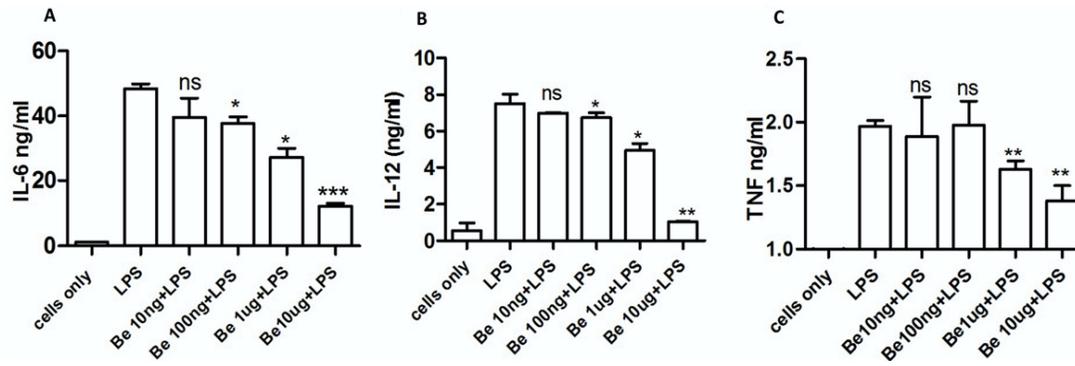
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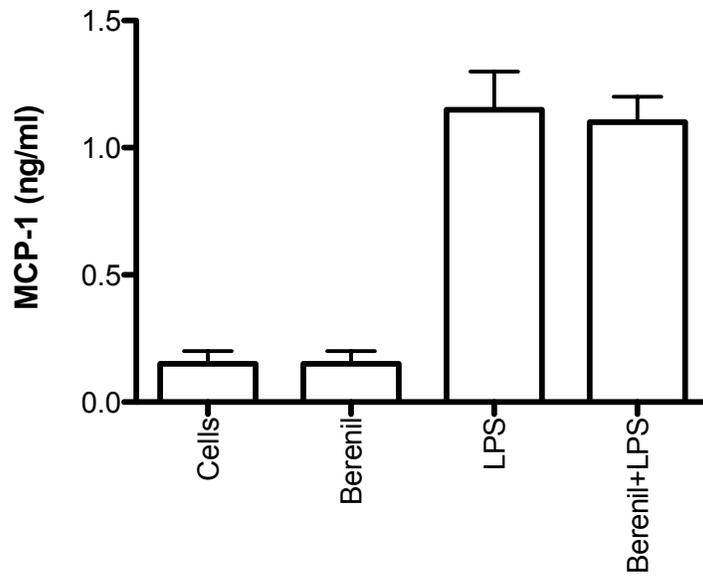
Appendices



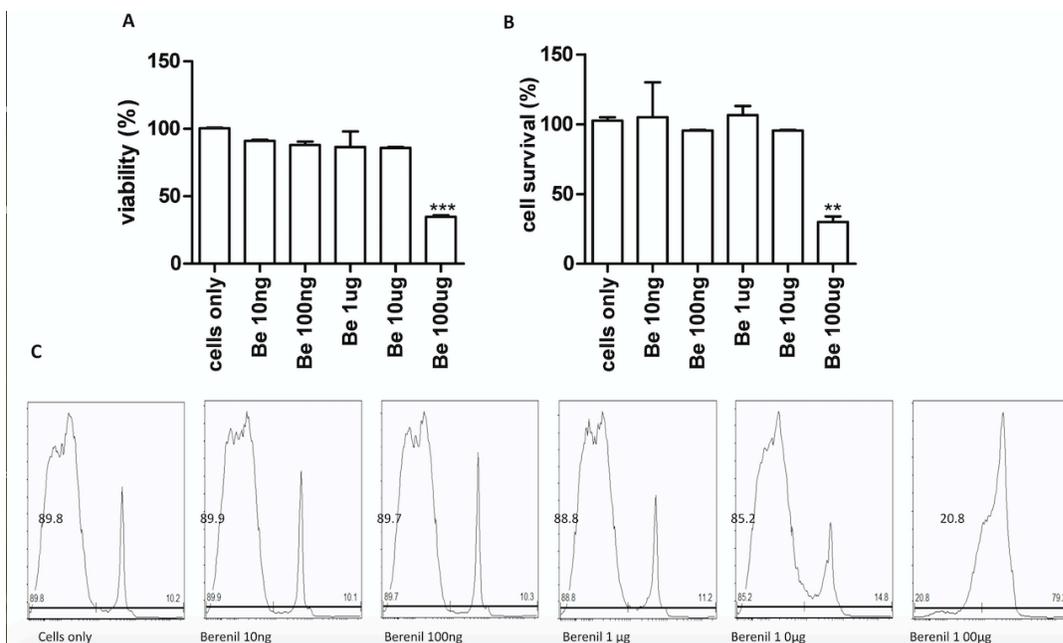
Appendix 1. Berenil treatment does not affect the frequency of CD4⁺CD25⁺FoxP3⁺ cells in the spleens of uninfected mice. Splenocytes from naïve (uninfected) BALB/c mice either treated or untreated with Berenil were stained directly *ex vivo* with fluorochrome-conjugated mAb against CD4, CD25 and Foxp3 and analyzed by flow cytometry. Presented are representative dot plots showing expression of CD25⁺ and Foxp3⁺ cells gated on CD4⁺ lymphocytes (C and F). The bar graphs represent the cumulative percentages of CD25⁺ and Foxp3⁺ cells (n=3 mice per group). The results presented are representative of 2 different experiments with similar results. Bars show mean +/-SEM.



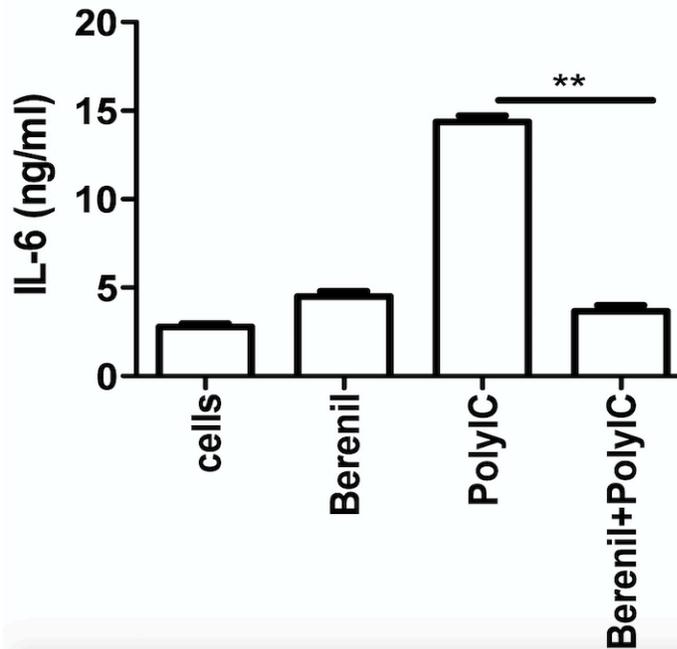
Appendix 2. Berenil pre-treatment downregulates LPS-induced cytokine production in ANA cells. ANA cells were pre-treated with Berenil (0.01-10 $\mu\text{g/ml}$) overnight, stimulated with LPS (1 $\mu\text{g/ml}$) for 12-16 hrs and the supernatant fluids were assayed for IL-6 (A), IL-12 (B) and TNF (C) by sandwich ELISA. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$



Appendix 3. Berenil pre-treatment did not affect LPS-induced MCP-1 production in ANA cells. ANA cells were pre-treated with Berenil (10 $\mu\text{g/ml}$) overnight, stimulated with LPS (1 $\mu\text{g/ml}$) for 12-16 hrs and the supernatant fluids were assayed for MCP-1 by sandwich ELISA.



Appendix 4. At less than 10 $\mu\text{g/ml}$, the drug was non-toxic to the cells. This was assayed by MTT (A), Trypan blue exclusion (B) and propidium iodide assay (C). The data presented are representative of 2 independent experiments with similar findings **, $p < 0.01$, ***, $p < 0.001$



Appendix 5. Berenil pre-treatment downregulates poly I:C-induced cytokine production in macrophages. BMDM were pretreated with Berenil (10 $\mu\text{g/ml}$) overnight, stimulated with poly I:C (5 $\mu\text{g/ml}$) for additional 12-16 hrs and the culture supernatant fluid was assayed for IL-6 by sandwich ELISA. The data presented are representative of 2 independent experiments with similar findings. **, $p < 0.01$.